Bisphenol A, genistein, daidzein and equol in urine

Matrix:	Urine
Hazardous substances:	Bisphenol A, genistein, daidzein
Analytical principle:	Capillary gas chromatography with mass selective detection (GC-MS)
Completed in:	November 2007

Overview of the parameters that can be determined with this method and the corresponding chemical substances:

Hazardous substance	CAS	Parameter	CAS
Genistein	446-72-0	Genistein	446-72-0
Daidzein	486-66-8	Daidzein	486-66-8
		Equol	531-95-3
Bisphenol A	80-05-7	Bisphenol A	80-05-7

Summary

The present method permits the quantitation of the isoflavones genistein and daidzein, of the daidzein metabolite equol and of bisphenol A in human urine.

For sample preparation, the internal standards are initially added to the urine samples. The analyte conjugates are then enzymatically hydrolysed, cleaned and enriched by means of solid phase extraction. After conversion to silyl ether derivatives the analytes are separated by gas chromatography and subjected to mass selective detection.

Genistein (GEN)

Within day precision:	Standard deviation (rel.)	s_w = 17% or 3%
	Prognostic range	u = 54% or 10%
	at a spiked concentration of 10 or 125 µg GEN per litre urine and where n = 4 determinations	

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Day to day precision:	Standard deviation (rel.)	s_w = 18%
	Prognostic range	u = 46%
	at a spiked concentration of 75 µg GEN per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate	r = 114% or 105%
	at a nominal concentration of 125 µg GEN per litre urine and where n = 4 determinations within day or from day to day	
Detection limit:	5 µg GEN per litre urine	
Quantitation limit:	18 µg GEN per litre urine	

Daidzein (DAI)

Within day precision:	Standard deviation (rel.)	s_w = 5% or 7%
	Prognostic range	u = 16% or 22%
	at a spiked concentration of 10 or 125 µg DAI per litre urine and where n = 4 determinations	
Day to day precision:	Standard deviation (rel.)	s_w = 9%
	Prognostic range	u = 23%
	at a spiked concentration of 75 µg DAI per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate	r = 109% or 104%
	at a nominal concentration of 125 µg DAI per litre urine and where n = 4 determinations within day or from day to day	
Detection limit:	4 µg DAI per litre urine	
Quantitation limit:	9 µg DAI per litre urine	

Equol (EQ)

Within day precision:	Standard deviation (rel.)	s_w = 6% or 5%
	Prognostic range	u = 19% or 16%
	at a spiked concentration of 10 or 125 µg EQ per litre urine and where n = 4 determinations	
Day to day precision:	Standard deviation (rel.)	s_w = 15%
	Prognostic range	u = 39%
	at a spiked concentration of 75 µg EQ per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate	r = 89% or 112%
	at a nominal concentration of 125 µg EQ per litre urine and where n = 4 determinations within day or from day to day	
Detection limit:	4 µg EQ per litre urine	
Quantitation limit:	11 µg EQ per litre urine	

Bisphenol A (BPA)

Within day precision:	Standard deviation (rel.)	s_w = 7% or 5%
	Prognostic range	u = 22% or 16%
	at a spiked concentration of 2.2 or 27.5 µg BPA per litre urine and where n = 4 determinations	
Day to day precision:	Standard deviation (rel.)	s_w = 10%
	Prognostic range	u = 26%
	at a spiked concentration of 16.5 µg BPA per litre urine and where n = 6 determinations	
Accuracy:	Recovery rate	r = 94% or 90%
	at a nominal concentration of 27.5 µg BPA per litre urine and where n = 4 determinations within day or from day to day	
Detection limit:	3 µg BPA per litre urine	
Quantitation limit:	7 µg BPA per litre urine	

Substances with estrogenic activity (genistein, daidzein, equol and bisphenol A)

Hormonally active substances or so-called "endocrine disruptors" are increasingly under discussion because exposure to these substances, especially in early life, can be accompanied by developmental and reproductive toxicity [1, 2]. In humans exposure usually takes place via food intake involving hormonally active substances of natural or anthropogenic origin [3, 4].

Phytoestrogens

Phytoestrogens are secondary plant ingredients including among other substances flavanones, flavones and isoflavones (see Figure 6 in the Appendix for basic structures). The best known representatives include the isoflavones daidzein (DAI) and genistein (GEN), which occur in many plants, but especially in soybean products [5]. Also the known phytoestrogens formononetin and biochanin A are enzymatically broken down to DAI and GEN (demethylation) by intestinal bacteria and liver microsomes [6, 7]. An overview of the phytoestrogen levels contained in foods is shown in Table 1.

The intake of DAI and GEN depends to a great extent on eating habits. The estimated daily intake of isoflavones is between 0.8 mg for a western style diet and 47 mg for a traditional Asian diet [14–17]. In more recent times, soy preparations are also gaining interest as dietary supplement providing relief in climacterium in the context of hormone replacement therapy. In such cases, isoflavone doses of 40 to 118 mg per day are recommended [18]. Through the enzyme systems in the gut flora of rodents and humans DAI is partially reduced to equol. Similarly, GEN is metabolised to 4-ethylphenol [19]. On account of its demonstrated xenoestrogenic structure, and as a metabolite of the isoflavone DAI, equol (EQ) has also been included in the method described below (see Figure 1 for structures).

Table 1 Phytoestrogen contents in foods.

Foods	Daidzein [µg/g]	Genistein [µg/g]	Formononetin [µg/g]	Biochanin A [µg/g]	Ref.
Alfalfa shoot	n.d.	n.d.	traces	traces	[8]
Bananas	n.d.	n.d.	–	–	[9]
Peas (green)	73	n.d.	traces	n.d.	[10]
Strawberries, fresh	0.045	0.457	–	–	[11]
Peanuts, roasted	0.037	0.172	–	–	[11]
Clover shoot	n.d.	4	22.8	4.4	[10]
Kidney beans	n.d.	n.d.	n.d.	4.1	[10]
Oranges	n.d.	n.d.	–	–	[11]
Beer (Pilsener Urquell)	0.0006	0.0018	0.004	0.0013	[12]
Soybeans	676–1007	612–1382	n.d.	n.d.	[10]
Soy protein, concentrate, washed in water	167–910	400–760	–	–	[13]
Soy shoot	138–225	113–305	–	–	[13]
Tofu	113	166	n.d.	n.d.	[10]

n. d. = not detected.

Bisphenol A

Bisphenol A (BPA) is a synthetic substance with endocrine activity and represents an important monomer in the production of epoxy resins, polycarbonates and other plastics. In 2009 the world production of BPA was 2.7 million tons, of which about 100 tons are released into the atmosphere per year [20]. This large-scale chemical product presents a hazard following exposure both in the production of the substance itself as well as in its further processing to polymers (polycarbonates and epoxy resins). On account of the endocrine activity of the substance a MAK value of 5 mg/m^3 (measured as inhalable fraction) was established for BPA [21]. Furthermore, a BLW (Biologischer Leitwert) for bisphenol A of 80 mg per litre urine has been established in 2006 [22].

BPA is found in many plastic flasks as well as in coatings of cans containing different foods. Through migration of BPA residual monomers into packed foods, the presence of BPA as contaminant in different beverages and foods can be demonstrated [23–25]. BPA has also been detected in the saliva of patients with tooth fillings in which the BPA monomer was used. The average exposure of an adult to BPA is about 30 µg per day (corresponding to approx. 0.5 µg/kg body weight) according to the European Commission [25].

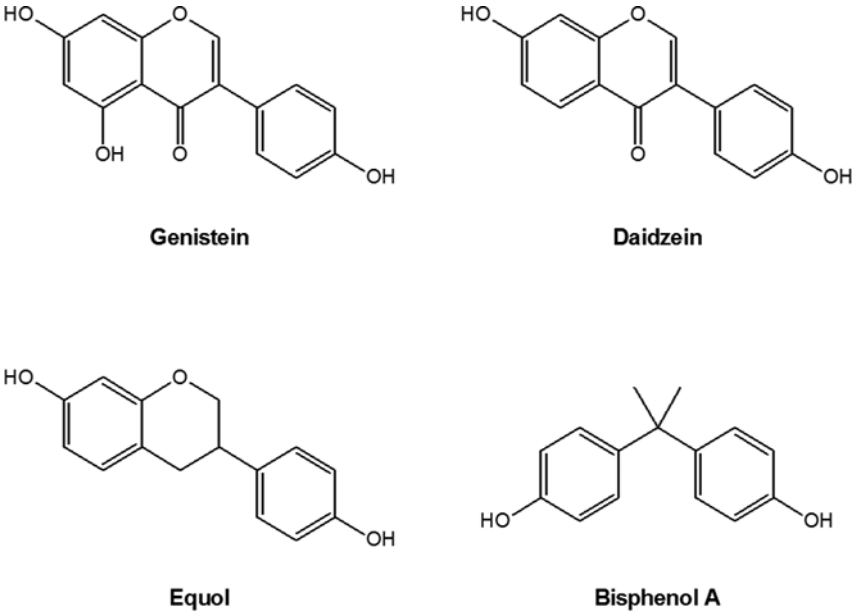

Fig. 1 Structures of the analysed substances with estrogenic activity.

These data form a frame of reference for the estimated daily intake of the estrogenically active isoflavones and BPA. However, as regards type and quantity, exposure can vary greatly. Although these exposure data form the basis for risk estimation, both the toxicodynamics (mode of action, hormonal activity) and the toxicokinetics (absorption, distribution, metabolism, elimination) as well as aspects of the affected endocrine system must also be taken into account for a toxicological evaluation of exposure to xenoestrogens [1, 26, 27]. The estrogenic activity of DAI, GEN and BPA was already analysed both *in vivo* and *in vitro* in many bioassays and was categorised as weak in comparison with the steroid estrogens [28–31]. Metabolism studies in rodents showed an extensive conjugation of isoflavones and BPA to polar metabolites by phase II enzymes [32–36]. In human studies a very similar metabolic pathway was observed [27, 37], which resulted in an effective elimination of the investigated xenoestrogens with elimination half-lives of 5-8 hours for DAI and GEN and less than 6 hours for BPA [38]. The compounds are mainly eliminated in urine in the form of polar metabolites. Exposure assessments are therefore possible from urinary biomonitoring studies. In the US-population, average urinary background levels of 68 µg DAI/g creatinine, 22 µg GEN/g creatinine and 8 µg EQ/g creatinine were found [39, 40].

The urinary analyte levels can provide useful information concerning external and internal exposure [34]. Whereas the lipophilic BPA is directly absorbed in the

small intestine, the absorption of the analysed isoflavones is apparently dependent on the form in which they are present in the food (aglycon or conjugate) [41, 42]. In soybeans, DAI and GEN are mainly available as glucosides, acetyl glucosides or malonyl glucosides, which must be deconjugated before absorption. The sugar residues can be cleaved by gastric acid, β-glucosidases and/or bacterial enzymes in the intestine [43]. Figure 2 shows the main pathways of isoflavone metabolism via phase II enzymes to glucuronides, sulfates or sulfato-glucuronides. The conjugates are rapidly eliminated with the urine.

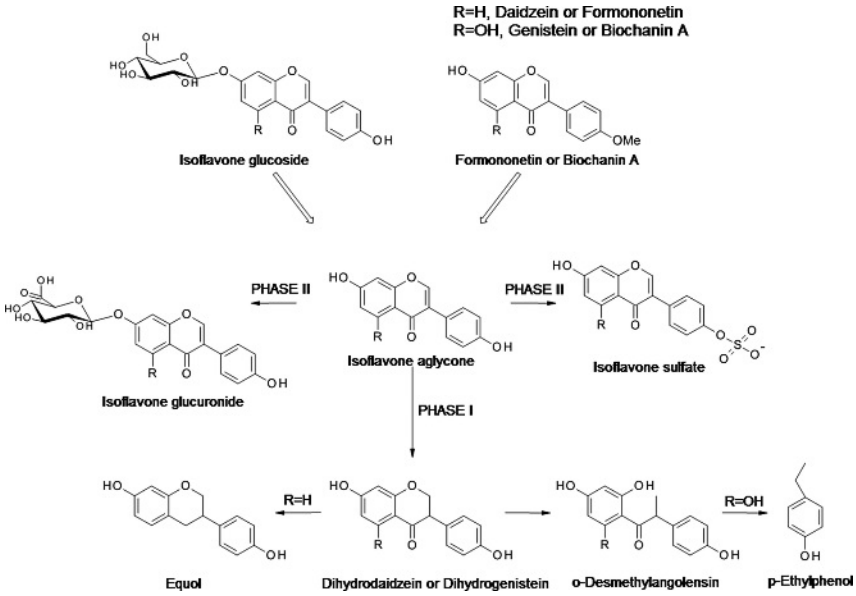

Fig. 2 Metabolism of isoflavones (only the main metabolites are shown).

Concerning hormonal activity, the conjugates of the isoflavones and of BPA are of minor importance, as they do not bind sufficiently to the intracellular estrogen receptor [35, 44–46]. In contrast, EQ shows *in vitro* an estrogenic activity similar to or even greater than that of DAI [47, 48]. 30–50% of the population are so-called equol eliminators, who are able to metabolise DAI to EQ [17, 42, 49, 50]. It is for this reason that EQ should always be determined when analysing isoflavones in the urine.

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Contents

1 General principles

To determine the isoflavones genistein and daidzein, the daidzein metabolite equol and bisphenol A in human urine samples, the internal standard substances are initially added to the samples. Then the urinary analyte conjugates are enzymatically hydrolysed, cleaned up and enriched using solid phase extraction. After conversion to silyl ether derivatives the analytes are separated by gas chromatography and subjected to mass selective detection.

2 Equipment, chemicals and solutions

2.1 Equipment and material

- Gas chromatograph (GC) with mass selective detector (MS), autosampler and data processing system (e.g. Agilent 6890N, Quadrupol MS 5973N, autosampler 7683, Software: Chemstation)
- Cooled injection system (e.g. Gerstel CIS 4 plus with Controller 505, Software: Master-software V1.8x)
- Gas chromatographic column: length: 15 m, inner diameter: 0.25 mm, stationary phase: 100% dimethylpolysiloxane, film thickness: 0.25 µm (e.g. DB-1-MS of J&W Scientific)
- pH meter (e.g. Metrohm)
- Thermostatic shaker (e.g. Thermomixer Comfort by Eppendorf)
- Vacuum station for solid phase extraction and dryer top (e.g. Phenomenex)
- Vacuum centrifuge (e.g. SPD 111V Speedvac, RVT 400 of Thermo Savant)
- Cooled vacuum trap (e.g. VN 100 vacuum Controller byThermo Electron)
- Membrane vacuum pump (e.g. MZ2C by Vaccubrand)
- Ultrasonic bath (e.g. Sonorex Rk 102H by Bandelin)
- Vortex mixer (e.g. Heidolph)
- Centrifuge (e.g. Eppendorf)
- Eppendorf tubes 0.5 mL, 1.5 mL and 2.0 mL (e.g. Eppendorf)
- Various glass beakers and glass flasks (e.g. Brand, Schott)
- C18 solid phase columns (e.g. Empore Extraction Cartrigde 4115 SD&HD of 3M)
- Lichrolut® RP 18 solid phase columns (500 mg, 3 mL polypropylene cartridges, e.g. Merck)
- Various volumetric flasks (e.g. Brand)
- Various pipettes with variable volumes (e.g. Eppendorf, Gilson)
- Pipette tips (e.g. Eppendorf, Sarstedt)
- 2 mL Sample vials made of borosilicate glass with 0.2 mL borosilicate glass in-

serts with polymer base, screw caps and silicon/polytetrafluoroethylene (PTFE) septa (e.g. VWR)
- Microlitre syringes 10 µL and 25 µL (e.g. Hamilton, SGE)

2.2 Chemicals

- Bisphenol A, 99% (e.g. Sigma Aldrich, No. 239658)
- $^{13}C_{12}$-Bisphenol A, 99% (e.g. Cambridge Isotope Laboratories, No. CLM-4325)
- Daidzein, ≥ 98% (e.g. Fluka, No. D7802)
- D_3-Daidzein (3′,5′,8-d_3), 97% (e.g. Cambridge Isotope Laboratories, No. DLM-4461)
- Equol, ≥ 99.0% (e.g. Fluka, No. 45405)
- Genistein, ≥ 98% (e.g. Fluka, No. G6649)
- D_4-Genistein (3′,5′,6,8-d_4), 95% (e.g. Cambridge Isotope Laboratories, No. DLM-4460)
- Acetonitrile, suprasolv (e.g. Merck, No. 100017)
- Ascorbic acid, ≥ 99% (e.g. Sigma, No. A5960)
- Glacial acetic acid p.a. (e.g. Merck, No. 100063)
- Ethyl acetate, for gas chromatography (e.g. Merck, No. 110972)
- Ethylene diamine tetraacetic acid (EDTA), anhydrous (e.g. Aldrich, No. 431788)
- n-Hexane, for gas chromatography (e.g. Merck, No. 104371)
- Methanol p.a. (e.g. Merck, No. 106018)
- Sodium acetate trihydrate p.a. (e.g. Merck, No. 106267)
- MTBSTFA with 1% TBDMCS (N-Methyl-N-tert-butyl dimethylsilyl trifluoroacetamide with 1% tert-butyl dimethylchlorosilane) (e.g. Aldrich, No. 375934)
- β-Glucuronidase (*Escherichia coli* K12) (e.g. Roche, No. 03707580001)
- Sulfatase (Abalone entrails, Type VIII) (e.g. Sigma-Aldrich, No. S9754)
- Water, bidistilled
- Nitrogen 5.0
- Helium 4.6

2.3 Solutions

- Hydrolysis buffer
  13.6 g sodium acetate trihydrate, 1.0 g ascorbic acid and 0.1 g EDTA are weighed into a 200 mL glass beaker and dissolved in approx. 70 mL bidist. water. The solution is then adjusted to pH = 5 with glacial acetic acid, transferred to a 100 mL volumetric flask and made up to the mark with bidist. water. The buffer should be colourless and stored at 4°C.
- β-Glucuronidase solution
  The β-glucuronidase (140 U/mL) dissolved in glycerol can be used directly. Isoflavones which may be present should be cleaned out before use for isoflavone analysis. For this purpose, the glucuronidase is passed through a Lichrolut RP18 column preconditioned with methanol and bidist. water.

- Sulfatase solution
  500 U sulfatase are dissolved in 5 mL bidist. water, divided into aliquots of 100 µL each in 0.5 mL Eppendorf tubes and stored at –20°C (100 U/mL) up to use. After preparation this solution is usable for up to one year when stored at –20°C. Once thawed, solutions should not be deep frozen again.
- Enzyme mixture (β-glucuronidase/sulfatase, 2:1)
  200 µL of the β-glucuronidase solution and 100 µL of the sulfatase solution are pipetted into a 0.5 mL Eppendorf tube and mixed thoroughly. The enzyme mixture must be freshly prepared for every analytical series.
- Wash solution (5% methanol in water)
  About 20 mL bidist. water are placed in a 50 mL volumetric flask, then 2.5 mL methanol are added and the flask is made up to the mark with bidist. water.
- Elution solution (ethyl acetate/acetonitrile, 1:1)
  Exactly 25 mL ethyl acetate are pipetted into a 50 mL volumetric flask and the flask is made up to the mark with acetonitrile.

2.4 Internal standards

- Stock solution of the internal standards (IS)
  200 µL d_3-daidzein (c = 60 µg/mL), 120 µL d_4-genistein (c = 100 µg/mL) and 60 µL $^{13}C_{12}$-bisphenol A (c = 100 µg/mL) are pipetted into a 2 mL glass vial and 220 µL acetonitrile are added. This solution contains 20 mg/L d_3-daidzein and 20 mg/L d_4-genistein as well as 10 mg/L $^{13}C_{12}$-bisphenol A.
- Working solution of the internal standards
  200 µL of the IS stock solution are pipetted into a 2 mL vial and mixed with 1800 µL bidist. water. The working solution contains 2 mg/L d_3-genistein and 2 mg/L d_4-daidzein as well as 1 mg/L $^{13}C_{12}$-bisphenol A.

2.5 Calibration standards

- Stock solution genistein (50 mg/L)
  Exactly 5 mg genistein are weighed into a 100 mL volumetric flask and made up to the mark with methanol.
- Stock solution daidzein (c = 50 mg/L)
  Exactly 5 mg daidzein are weighed into a 100 mL volumetric flask and made up to the mark with methanol.
- Stock solution equol (c = 50 mg/L)
  Exactly 1 mg equol is weighed into a 20 mL volumetric flask and made up to the mark with methanol.
- Stock solution bisphenol A (c = 2.2 g/L)
  Exactly 110 mg BPA are placed in a 50 mL volumetric flask and made up to the mark with methanol.

The stability of the stock solutions was not checked. However, no relevant changes in calibrations which were prepared from the same stock solution were found over a period of > 3 months.

Dilutions for calibration

- Bisphenol A predilution (c = 22 mg/L)
  1 mL of the BPA stock solution is placed in a 100 mL volumetric flask and made up to the mark with methanol.
- Working solution I (c = 5 mg/L or 1.1 mg/L)
  1 mL of each of the stock solutions of equol, daidzein and genistein as well as 500 µL of the bisphenol A predilution are pipetted into a 10 mL volumetric flask and made up to the mark with bidist. water. Working solution I contains 5 mg/L each of EQ, DAI and GEN as well as 1.1 mg/L of BPA.
- Working solution II (c = 0.5 mg/L or 0.11 mg/L)
  1 mL of working solution I is pipetted into a 10 mL volumetric flask and made up to the mark with bidist. water. Working solution II contains 0.5 mg/L each of EQ, DAI and GEN as well as 0.11 mg/L of BPA.

Calibration

The calibration standards are prepared in 1.5 mL Eppendorf tubes according to the pipetting scheme in Table 2. For calibration, isoflavone free pooled urine should be used which has been obtained from volunteers who have been on an isoflavone free diet (in particular no soybean products) for at least 2.5 days. As far as possible, the blank value of the prepared calibration series should show no quantifiable analyte signals.

Table 2 Pipetting scheme for the preparation of the calibration standards.

Calibration standard	Spiked analyte level [µg/L]		Pooled urine [µL]	Working soln. I [µL]	Working soln. II [µL]	IS Working soln. [µL]
	EQ, DAI, GEN	BPA				
Blind value	–	–	200	–	–	–
K0	0	0	200	–	–	8
K1	12.5	2.8	200	–	5	8
K2	25.0	5.5	200	–	10	8
K3	50.0	11.0	200	–	20	8
K4	100	22.0	200	4	–	8
K5	250	55.0	200	10	–	8

3 Specimen collection and sample preparation

3.1 Specimen collection

No fixed sampling time is required for the determination of urinary background levels. The urine samples are kept in polyethylene containers at –20°C until preparation.

3.2 Sample preparation

The urine sample is thawed before analysis and equilibrated to room temperature. A 200 µL aliquot of the sample is transferred to a 1.5 mL Eppendorf tube, to which 8 µL of the internal standard working solution is added. Subsequently, the mixture is shaken thoroughly.

Hydrolysis

10 µL of the hydrolysis buffer and 12 µL of the enzyme mixture are added to the sample, which is then incubated overnight (17 h) in a thermostatic shaker at 37°C using the maximum mixing frequency.

Solid phase extraction

The SPE C18 columns are conditioned using 300 µL methanol and 300 µL bidist. water that are subsequently transferred and passed through each column using the vaccum pump. Care must be taken that the SPE columns never completely run dry!

The sample hydrolysed overnight is centrifuged at 16000 *g* for 1 min. Then the complete sample solution is transferred to the conditioned SPE column using a 300 µL pipette and passed through the SPE column under vacuum. Care must again be taken that the SPE column does not completely run dry. The column is then washed with 300 µL of the wash solution (5% methanol in water) and then dried under vacuum for 10 min. Elution of the analytes is carried out with 300 µL of the elution solution (ethyl acetate / acetonitrile) in a new 1.5 mL Eppendorf tube. For this purpose the elution solution is passed slowly through the SPE column under slight vacuum. Though a formation of two phases in the Eppendorf tube indicates insufficient drying, this has no negative influence on further sample preparation. The sample is then evaporated to dryness using a vacuum centrifuge at 40°C (approx. 1 up to 2 h).

Derivatisation (silylation)

50 µL of the silylation agent (MTBSTFA with 1% TBDMCS) is added to the dried sample, which is then incubated for about 2 min in the ultrasonic bath and briefly vortexed. The sample is then incubated for 30 min at 75°C in the thermostatic shaker (maximum mixing frequency). After cooling to room temperature the sample solution is evaporated to dryness in a stream of nitrogen under gentle heating (40 °C).

After addition of 70 µL n-hexane the sample is incubated in the ultrasonic bath for 1 min, briefly vortexed and then centrifuged at 16000 *g* for 1 min. The super-

natant is then transferred with a pipette into a 2 mL sample vial with a micro-insert and is immediately analysed.

4 Operational parameters

The analysis of the samples prepared according to Section 3 is carried out using a GC-MS unit (e.g. Agilent GC 6890 with Quadrupol MS 5973N and Gerstel MPS2) with following system parameters:

4.1 Operational parameters for gas chromatography

Capillary column:	Material:	Fused Silica
	Stationary phase:	DB-1ms
	Length:	15 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Temperature:	Column:	2 min at 160°C; then increase at 30°C/min to 310°C; 5 min at final temperature
	Injector:	0.08 min at 25°C; then increase at 12°C/s to 320°C; hold for 2 min, then increase at 12°C/s to 400°C
	Transfer line:	280°C
Injection:	Liner:	standard liner baffled
	Injection method:	Cooled injection system (CIS 4 plus): 20 µL solvent vent injection, 200 mL/min Helium; solvent vent (in the split vent: 100 mL/min for complete liner heating)
	Injection velocity:	4 µL/s
Carrier gas:	Helium 4.6	
	Constant flow:	2.0 mL/min

4.2 Mass spectrometric parameters

Ionisation type:	Electron impact ionisation (EI)	
Temperatures:	Source:	230°C
	Quadrupol:	150°C
Ionisation energy:	70 eV	

All other parameters must be optimised in accordance with the manufacturer's instructions.

5 Analytical determination

For an additional enrichment of the analytes a cooled injection system is used for gas chromatographic analysis in which 20 µL sample solution are injected and reduced by evaporation. A blank value and a quality control sample as well as a high and a low calibration standard are included for control with each analytical series.

The time profiles of the ion traces listed in Table 3 are recorded in the single ion monitoring (SIM) mode.

The retention times given in Table 3 are intended to be a rough guide only. Users of the method must ensure proper separation performance of the analytical column they use and of the resulting retention behaviour of the analyte.

Table 3 Retention times and recorded masses.

Parameter	Retention time [min]	Quantifier [m/z]	Qualifier [m/z]
Bisphenol A	5.1	441	213, 456
$^{13}C_{12}$-Bisphenol A	5.1	453	225, 468
Equol	6.0	470	234
Daidzein	6.8	425	482
D_3-Daidzein	6.8	428	485
Genistein	7.2	555	–
D_4-Genistein	7.2	558	–

In Figure 3 the chromatogram of a calibration standard is shown which was produced using an injection volume of 20 µL in the solvent vent (cooled injection system). Figure 4 shows a chromatogram of the standard solutions of BPA, daidzein and genistein together with the corresponding internal standards.

6 Calibration

The calibration standards are prepared according to the instructions in Section 3.2 and analysed using GC-MS according to Sections 4 and 5. The calibration graphs are obtained by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the calibration standards. As no isotopically labelled standard is available for equol, $^{13}C_{12}$-BPA is used as an internal standard for both, equol and bisphenol A. $^{13}C_{12}$-BPA showed better results for equol in comparison to the isotopically labelled isoflavones. For routine analysis it is recommended to carry out a complete calibration once per week. Otherwise a blank value, a quality control sample as well as a high and a low calibration standard should be included within each analytical series. The calibration graph is linear between the detection limits and concentrations of 250 µg DAI, 250 µg GEN,

250 µg EQ or 110 µg BPA each per litre urine. Figure 5 shows an example of the calibration graphs for the four analytes.

7 Calculation of the analytical result

To determine the analyte concentration in a sample, the peak area of each analyte is divided by the peak area of the respective internal standard. The ratio thus obtained, is inserted into the corresponding calibration graph (see Section 6). The analyte concentration in µg per litre urine is obtained.

8 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) [51–53]. To obtain reliable results it is recommended to perform double determinations.

To check the precision a spiked urine sample is analysed within each analytical series, which contains a constant and known concentration level of the analytes. As a material for quality control is not commercially available, the control material must be prepared in the laboratory by spiking of isoflavone free pooled urine (see Section 2.5). The quality control material is then divided into aliquots and stored at –20°C. The analyte concentration level of the control material should lie within the relevant concentration range. The nominal value and the tolerance ranges of the quality control material are determined in a pre-analytical period [53].

With the analysis of two calibration standards as well as of one reagent blank value per day of analysis, it is not necessary to include a complete calibration with each analytical series. A new calibration curve should be plotted when the results of quality control indicate systematic deviations of more than 15–20%. In any case, a blank value should be included within each analytical series to detect possible contaminations.

9 Evaluation of the method

9.1 Precision

To determine the within day precision, spiked urine samples with low and high analyte levels were analysed four times in a row. The precision data thus obtained are listed in Table 4.

To determine the precision from day to day, urine samples spiked with standard solutions were prepared and analysed on six different days. The results are listed in Table 5.

Table 4 Within day precision (n = 4).

Parameter	Spiked analyte level [µg/L]		Standard deviation (rel.) [%]		Prognostic range [%]	
	Urine 1	Urine 2	Urine 1	Urine 2	Urine 1	Urine 2
Genistein	10	125	17	3	54	10
Daidzein	10	125	5	7	16	22
Equol	10	125	6	5	19	16
Bisphenol A	2.2*	27.5	7	5	22	16

* Although this concentration is below the given detection limit, it can nevertheless be determined with a certain reliability. However, more extensive deviations were occasionally found in the low concentration range (see Section 9.5).

Table 5 Day to day precision (n = 6).

Parameter	Spiked analyte level [µg/L]	Standard deviation (rel.) [%]	Prognostic range [%]
Genistein	75	18	46
Daidzein	75	9	23
Equol	75	15	39
Bisphenol A	16.5	10	26

9.2 Accuracy

Recovery experiments with spiked urine samples were carried out to determine the accuracy of the method. For this purpose pooled urine was spiked with defined analyte levels, processed four times and analysed. The determinations were carried out both within day as well as from day to day. The mean relative recovery rates thus obtained are shown in Table 6.

Table 6 Relative recovery rates within day and from day to day (n = 4).

Parameter	Spiked analyte level [µg/L]	Mean relative recovery [%] (n = 4)	
		Within day	From day to day
Genistein	10	119	–
	75	–	126
	125	114	105
Daidzein	10	108	–
	75	–	127
	125	109	104
Equol	10	104	–
	75	–	90
	125	89	112
Bisphenol A	16.5	–	119
	27.5	94	90

9.3 Detection limits and quantitation limits

The detection and quantitation limits were calculated in accordance with DIN method 32645 [German Industrial Standard, *Deutsche Industrie Norm*] (calibration graph method). For this purpose, three independent calibration graphs with 10 calibration points per analyte were performed. The results are listed in Table 7.

Table 7 Detection and quantitation limits, calculated according to DIN 32645.

Parameter	Detection limit [µg/L]	Quantitation limit [µg/L]
Genistein	5	18
Daidzein	4	9
Equol	4	11
Bisphenol A	3	7

9.4 Validation data of the alternative method

As an alternative for direct splitless injection of 20 µL of the prepared sample solution (see Section 3.2) the method can also be carried out without the use of a cooled injection system. For this only 2 µL of the sample solution are injected directly (splitless) into the GC-MS system. This procedure was applied as part of the successful examination of the method. The validation data thus obtained are described in Table 8. In spite of the 10-fold lower injection volume the detection limits using the alternative injection method are only 2–4 times higher.

Table 8 Validation data when using an injection volume of 2 µL.

Parameter	Spiked analyte level [µg/L]	BPA	Equol	Daidzein	Genistein
Detection limit [µg/L]		5	15	11	9
Quantitation limit [µg/L]		9	29	26	20
Within day precision	50	8	12	8	11
[%, n = 6]	125	5	9	5	7
Day to day precision	50	7	13	9	12
[%, n = 4]	125	8	9	8	9
Rel. recovery rate (Accuracy)	30	109 ± 10	102 ± 6	110 ± 5	115 ± 7
[%, n = 4]	50	101 ± 4	102 ± 4	100 ± 5	99 ±5
	125	100 ± 4	100 ± 2	99 ± 4	98 ± 4
Absolute recovery after extraction [%, n = 6]		81 ± 2	88 ± 4	85 ± 5	90 ± 2

9.5 Sources of error

BPA is ubiquitous and may also be found in house dust. During sample preparation special cleanliness must be ensured. Insufficiently washed containers may otherwise produce a considerable blank value for BPA. The bidistilled water used may also contain BPA. Therefore the water used should previously be cleaned by passing it through a Lichrolut RP 18 cartridge preconditioned with methanol. A reagent blank value is therefore to be included in every analytical series to monitor the blank value.

The fragmentation pattern of the analytes in the mass spectrometer is greatly influenced by the pH value of the sample. Therefore, as part of the sample preparation, the urine samples are buffered to avoid interferences. The analysis of highly concentrated urine samples, especially urine samples from volunteers whose diet has a high soy content (e.g. Asian cuisine) can lead to carry-over contaminations when using the cooled injection system. In this case the heating of the liner should be extended or modified or alternatively 2 µL of the sample should be injected (see also Section 10).

In order to minimise the memory effect, a regular injection of pure n-hexane between the samples is recommended, especially after injection of highly concentrated samples. If the linear range of the method is exceeded, the samples should be diluted and processed again.

10 Discussion of the method

The present method was developed based on the studies by Liggins et al., Setchell et al. and Moors et al. [54–56] and allows a sensitive determination of substances with estrogenic activity, like genistein, daidzein, equol and bisphenol A. As the listed substances are mainly eliminated with the urine in the form of conjugates, urinary determination is carried out after hydrolysis, in which glucuronides and sulfates are cleaved. In order to guarantee a sensitive detection using GC-MS, the determination of the analytes is carried out after derivatisation of the free hydroxyl groups. In Figure 7 the applied silylation reaction is described using GEN as example.

The importance of the method lies mainly in the field of environmental medicine, as the phytoestrogens DAI and GEN are almost exclusively taken in via food. The large-scale chemical product bisphenol A, to which an estrogenic activity is also attributed, is considered to be relevant for occupational medicine as well.

The simultaneous detection of these four important hormonally active substances makes the analytical method applicable and useful for both, occupational and environmental medicine.

In the present method, sensitivity is increased when using a cooled injection system, whereby 20 µL of the sample solution is injected and reduced by evapora-

tion with the aid of a temperature program in the injector block. Alternatively, it is also possible to apply the method by injecting 2 µL of the prepared sample directly into the GC-MS unit. The detection limits are 2 to 4 times higher when using this alternative method. The linear working range of the method is, however, limited by the peak detector capacity; in the case of the Agilent MSD 5973N mass spectrometer used here, this is limited to 130 million area units. With a volume of 20 µL used in the cooled injection mode this limit was approximately 300 to 400 µg analyte per litre urine. Urine samples with such high isoflavone concentrations are, for example, to be expected in persons with a high soy intake. However, with a sample dilution, it is also possible to detect the mentioned analytes in highly concentrated samples.

Instruments used:

- Gas chromatograph (GC 6890, Agilent, USA) with autosampler (MPS2, Gerstel, Germany) and with a Quadrupole-mass spectrometer (5973N, Agilent, USA).

11 References

1 National Research Council: Hormonally Active Agents in the Environment. National Academy Press, Washington DC (1999).
2 G.H. Degen and H.M. Bolt: Endocrine disruptors: Update on xenoestrogens. Int. Arch. Occup. Environ. Health 73, 433–441 (2000).
3 S. Fritsche and H. Steinhart: Occurrence of hormonally active compounds in food: a review. Eur. Food Res. Technol. 209, 153–179 (1999).
4 G.H. Degen: Endokrine Disruptoren in Lebensmitteln. Bundesgesundheitsblatt 9, 848-856 (2004).
5 K. Reinli and G. Block: Phytoestrogen content of foods – a compendium of literature values. Nutr. Cancer 26, 123–148 (1996).
6 W.H. Tolleson, D.R. Doerge, M.I. Churchwell, M.M. Marques, D.W. Roberts: Metabolism of biochanin A and formononetin by human liver microsomes in vitro. J. Agric. Food Chem. 50, 4783–4790 (2002).
7 H. Hur and F. Rafii: Biotransformation of the isoflavonoids biochanin A, formononetin, and glycitein by Eubacterium limosum. FEMS Microbiol. Lett. 192, 21–25 (2000).
8 H. Saloniemi, K. Wähälä, P. Nykänen-Kurki, K. Kallela, I. Saastamoinen: Phytoestrogen content and estrogenic effect of legume fodder. Proc. Soc. Exp. Biol. Med. 208, 13–17 (1995).
9 W. Mazur: Phytoestrogen content in foods. Baillieres Clin. Endocrinol. Metab. 12, 729–742 (1998).
10 A.A. Franke, L.J. Custer, C.M. Cerna, K.K. Narala: Quantitation of phytoestrogens in legumes by HPLC. J. Agric. Food Chem. 42, 1905–1913 (1994).
11 J. Liggins, L.J. Bluck, S. Runswick, C. Atkinson, W.A. Coward, S.A. Bingham: Daidzein and genistein content of fruits and nuts. J. Nutr. Biochem. 11, 326–331 (2000).
12 O. Lapcik, M. Hill, R. Hampl, K. Wähälä, H. Adlercreutz: Identification of isoflavonoids in beer. Steroids 63, 14–20 (1998).

13 U.S. Department of Agriculture, Agricultural Research Service: USDA-Iowa State University Database on the Isoflavone Content of Foods. Release 1.3 (2002). URL: http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html visited on 06.06.2011.
14 L.K. Boker, Y.T. Van der Schouw, M.J. De Kleijn, P.F. Jacques, D.E. Grobbee, P.H. Peeters: Intake of dietary phytoestrogens by Dutch women. J. Nutr. 132, 1319–1328 (2002).
15 M.J. De Kleijn, Y.T. Van der Schouw, P.W. Wilson, H. Adlercreutz, W. Mazur, D.E. Grobbee, P.F. Jaques: Intake of dietary phytoestrogens is low in postmenopausal women in the United States: the Framingham study (1–4). J. Nutr. 131, 1826–1832 (2001).
16 P.L. Horn-Ross, S. Barnes, M. Lee, L. Coward, J.E. Mandel, J. Koo, E.M. John, M. Smith: Assessing phytoestrogen exposure in epidemiologic studies: development of a database (United States). Cancer Causes Control 11, 289–298 (2000).
17 Y. Arai, M. Uehara, Y. Sato, M. Kimira, A. Eboshida, H. Adlercreutz, S. Watanabe: Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. J. Epidemiol. 10, 127–135 (2000).
18 T. Usui: Pharmaceutical prospects on phytoestrogens. Endocr. J. 53, 7–20 (2006).
19 E. Bowey, H. Adlercreutz, I. Rowland: Metabolism of isoflavones and lignans by the gut microflora: a study in germfree and human flora associated rats. Food Chem. Toxicol. 41, 631–636 (2003).
20 L.N. Vandenberg, M.V. Maffini, C. Sonnenschein, B.S. Rubin, A.M. Soto: Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. Endocrine Reviews 30, 75–95 (2009).
21 H. Greim (ed.): Bisphenol A. MAK Value Documentations. Volume 13, VCH-Verlag, Weinheim (1999).
22 H. Greim and H. Drexler (eds.): Bisphenol A. Biologische Arbeitsstoff-Toleranz-Werte (BAT-Werte) und Expositionsäquivalente für krebserzeugende Arbeitsstoffe (EKA) und Biologische Leitwerte (BLW). 14th issue, Wiley-VCH-Verlag, Weinheim (2007).
23 A. Goodson, W. Summerfield, I. Cooper: Survey of bisphenol A and bisphenol F in canned foods. Food Addit. Contam. 19, 796–802 (2002).
24 B.L. Tan and A.M. Mustafa: Leaching of bisphenol A from new and old babies' bottles, and new babies' feeding teats. Asia Pac. J. Public Health 15, 118-123 (2003).
25 Scientific Committee on Food: Opinion of the Scientific Committee on Food on Bisphenol A (expressed on 17 April 2002). European Commission, URL: http://ec.europa.eu/food/fs/sc/scf/out128_en.pdf, visited on 06.06.2011.
26 G.H. Degen, H. Foth, R. Kahl, H. Kappus, H.G. Neumann, F. Oesch, R. Schulte-Hermann: Hormonell aktive Substanzen in der Umwelt: Xenoöstrogene. Stellungnahme der Beratungskommission der Sektion Toxikologie der DGPT. Umweltmed. Forsch. Praxis 4, 367–374 (1999).
27 G.H. Degen, P. Janning, J. Wittsiepe, A. Upmeier, H.M. Bolt: Integration of mechanistic data in the toxicological evaluation of endocrine modulators. Toxicol. Lett. 127, 225–237 (2002).
28 G. Eisenbrand, B. Mußler, G.H. Degen: Kombinationswirkungen hormonartig wirkender Chemikalien. In: H.M. Bolt, B. Griefahn, H. Heuer, W. Laurig (Hrsg): Arbeitsphysiologie heute. Band 2. IfADo, Dortmund, S. 27–39 (2000).
29 P. Diel, T. Schulz, K. Smolnikar, E. Strunck, G. Vollmer, H. Michna: Ability of xeno- and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotrophic activity. J. Steroid Biochem. Mol. Biol. 73, 1–10 (2000).
30 P. Diel, S. Schmidt, G. Vollmer, P. Janning, A. Upmeier, H. Michna, H.M. Bolt, G.H. Degen: Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch. Toxicol. 78, 183–193 (2004).

31 S. Schmidt, G.H. Degen, J. Seibel, T. Hertrampf, G. Vollmer, P. Diel: Hormonal activity of combinations of genistein, bisphenol A and 17ß-estradiol in the female Wistar rat. Arch. Toxicol. 80, 839–845 (2006).

32 P. Janning, U.S. Schuhmacher, A. Upmeier, P. Diel, H. Michna, G.H. Degen, H.M. Bolt: Toxicokinetics of the phytoestrogen daidzein in female DA/Han rats. Arch. Toxicol. 74, 421–430 (2000).

33 T. Bayer, T. Colnot, W. Dekant: Disposition and biotransformation of the estrogenic isoflavone daidzein in rats. Toxicol. Sci. 62, 205–211 (2001).

34 G.H. Degen, P. Janning, P. Diel, H. Michna, H.M. Bolt: Transplacental transfer of the phytoestrogen daidzein in DA/Han rats. Arch. Toxicol. 76, 23–29 (2002).

35 R.W. Snyder, S.C. Maness, K.W. Gaido, F. Welsch, S.C. Sumner, T.R. Fennell: Metabolism and disposition of bisphenol A in female rats. Toxicol. Appl. Pharmacol. 168, 225–234 (2000).

36 S. Moors, P. Diel, G.H. Degen: Toxicokinetics of bisphenol A in pregnant DA/Han rats after single i.v. application. Arch. Toxicol. 80, 647–655 (2006).

37 J.W. Lampe: Isoflavonoid and lignan phytoestrogens as dietary biomarkers. J. Nutr. 133, 956S–964S (2003).

38 W. Völkel, T. Colnot, G.A. Csanády, J.G. Filser, W. Dekant: Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem. Res. Toxicol. 15, 1281–1287 (2002).

39 L. Valentín-Blasini, M.A. Sadowski, D. Walden, L. Caltabiano, L.L. Needham, D.B. Barr: Urinary phytoestrogen concentrations in the U.S. population (1999–2000). J. Expo. Anal. Environ. Epidemiol. 15, 509–523 (2005).

40 Centers for Disease Control and Prevention (CDC): Third National Report on Human Exposure to Environmental Chemicals. CDC, Atlanta (GA), S. 285–306 (2005).

41 K.D. Setchell, N.M. Brown, L. Zimmer-Nechemias, W.T. Brashear, B.E. Wolfe, A.S. Kirschner, J.E. Heubi: Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am. J. Clin. Nutr. 76, 447–453 (2002).

42 M.S. Faughnan, A. Hawdon, E. Ah-Singh, J. Brown, D.J. Millward, A. Cassidy: Urinary isoflavone kinetics: the effect of age, gender, food matrix and chemical composition. Br. J. Nutr. 91, 567–574 (2004).

43 A.J. Day, M.S. DuPont, S. Ridley, M. Rhodes, M.J. Rhodes, M.R. Morgan, G. Williamson: Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett. 436, 71–75 (1998).

44 J.B. Matthews, K. Twomey, T.R. Zacharewski: In vitro and in vivo interactions of bisphenol A and its metabolite, bisphenol A glucuronide, with estrogen receptor alpha and beta. Chem. Res. Toxicol. 14, 149–157 (2001).

45 E. Federici, A. Touché, S. Choquart, O. Avanti, L. Fay, E. Offord, D. Courtois: High isoflavone content and estrogenic activity of 25 year-old Glycine max tissue cultures. Phytochemistry 64, 717–724 (2003).

46 Y. Zhang, T.T. Song, J.E. Cunnick, P.A. Murphy, S. Hendrich: Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. J. Nutr. 129, 399–405 (1999).

47 S.O. Mueller, S. Simon, K. Chae, M. Metzler, K.S. Korach: Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor alpha (ERalpha) and ERbeta in human cells. Toxicol. Sci. 80, 14–25 (2004).

48 N. Sathyamoorthy and T.T. Wang: Differential effects of dietary phytoestrogens daidzein and equol on human breast cancer MCF-7 cells. Eur. J. Cancer 33, 2384–2389 (1997).

49 I.R. Rowland, H. Wiseman, T.A. Sanders, H. Adlercreutz, E.A. Bowey: Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr. Cancer 36, 27–32 (2000).

50 P.B. Grace, J.I. Taylor, N.P. Botting, T. Fryatt, M.F. Oldfield, S.A. Bingham: Quantification of isoflavones and lignans in urine using gas chromatography/mass spectrometry, Anal. Biochem. 315, 114–121 (2003).

51 Bundesärztekammer: Qualitätssicherung der quantitativen Bestimmungen im Laboratorium. Neue Richtlinien der Bundesärztekammer. Dt. Ärztebl. 85, A699-A712 (1988).

52 Bundesärztekammer: Ergänzung der "Richtlinien der Bundesärztekammer zur Qualitätssicherung in medizinischen Laboratorien". Dt. Ärztebl. 91, C159–C161 (1994).

53 J. Angerer, T. Göen, G. Lehnert: Mindestanforderungen an die Qualität von umweltmedizinisch-toxikologischen Analysen. Umweltmed. Forsch. Prax. 3, 307–312 (1998).

54 J. Liggins, L. Bluck, W.A. Coward, S.A. Bingham: A simple method for the extraction and quantification of daidzein and genistein in food using gas chromatography mass spectrometry. Biochem. Soc. Trans. 26, S87 (1998).

55 K.D. Setchell, N.M. Brown, P. Desai, L. Zimmer-Nechemias, B.E. Wolfe, W.T. Brashear, A. S. Kirschner, A. Cassidy, J.E. Heubi: Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J. Nutr. 131, 1362S–1375S (2001).

56 S. Moors, M. Blaszkewicz, H.M. Bolt, G.H. Degen: Simultaneous determination of daidzein, equol, genistein and bisphenol A in human urine by a fast and simple method using SPE and GC-MS. Mol. Nutr. Food Res. 51, 787–798 (2007).

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12 Appendix

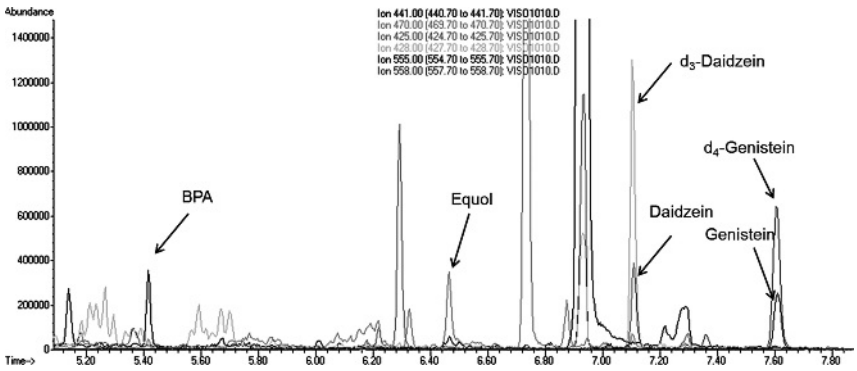


Fig. 3 Chromatogram of a calibration standard in urine.

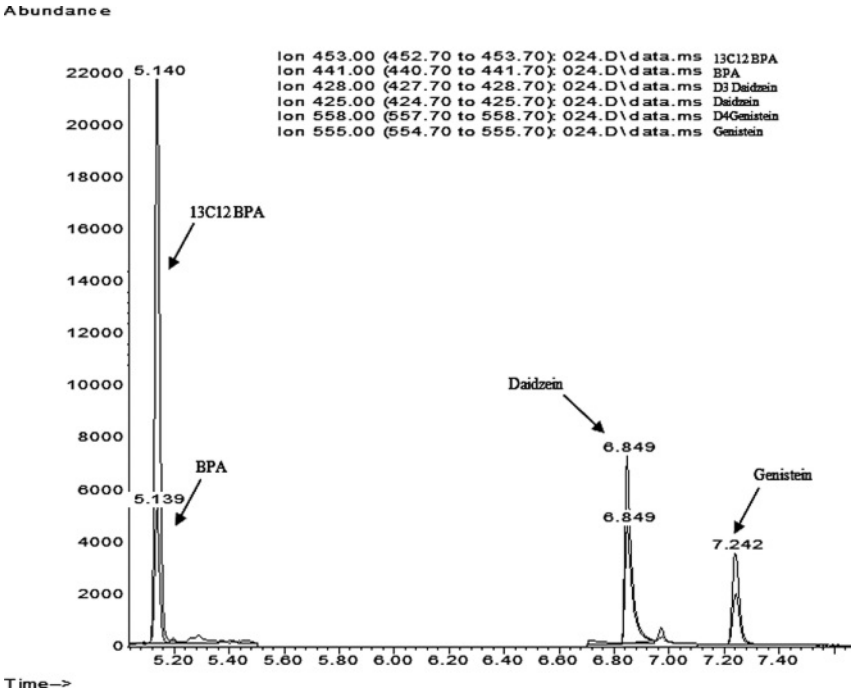


Fig. 4 Chromatogram of the analytes BPA, daidzein and genistein with the internal standards.

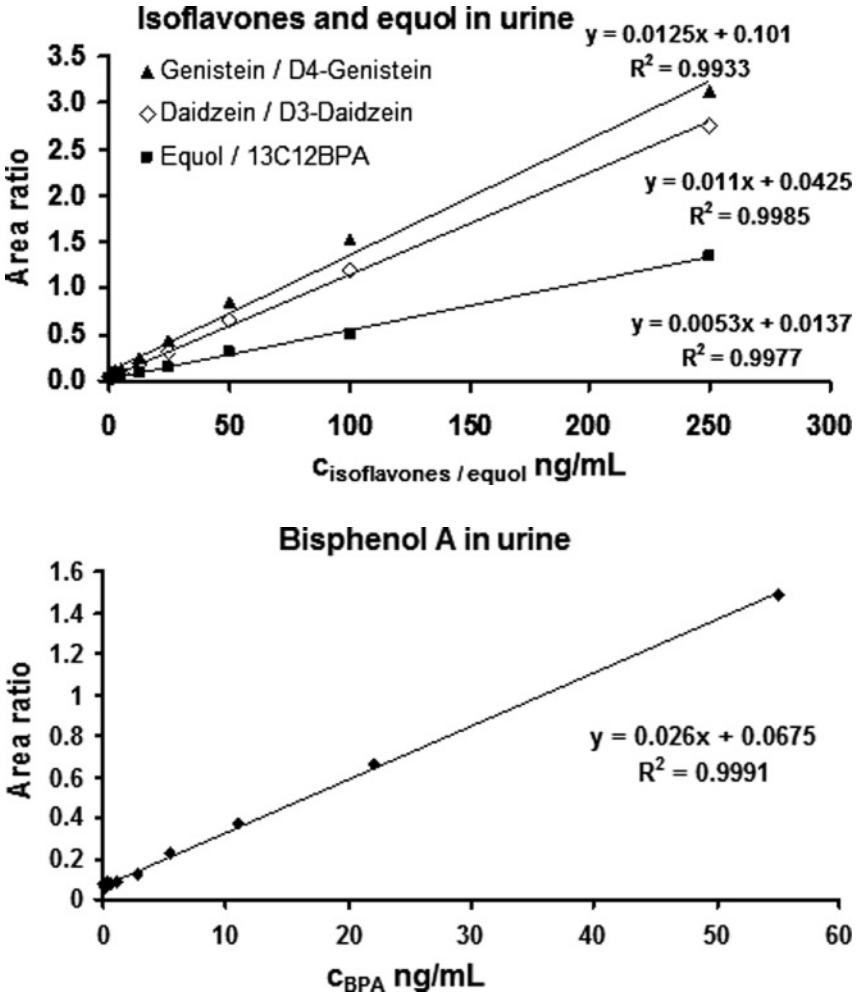
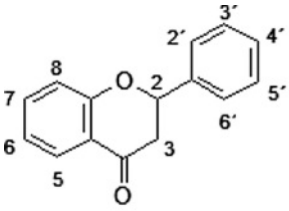
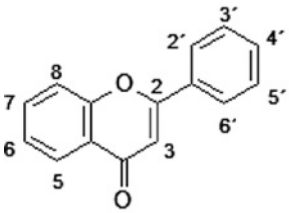


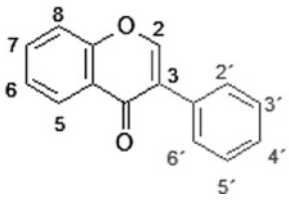
Fig. 5 Calibration functions: A: isoflavones and equol, B: bisphenol A.



Flavanones
Naringenin: 4',5,7-Trihydroxyflavanone
Sakuranetin: 4',5-Dihydroxy-7-methoxyflavanone



Flavones
Primuletin: 5-Hydroxyflavone
Chrysin: 5,7-Hydroxyflavone
Apigenin: 4',5,7-Hydroxyflavone
Kaempferol: 3,4',5,7-Tetrahydroxyflavone



Isoflavones
Daidzein: 4',7-Dihydroxyisoflavone
Formononetin: 7-Hydroxy-4'-methoxyisoflavone
Genistein: 4',5,7-Trihydroxyisoflavone
Biochanin A: 5,7-Dihydroxy-4'-methoxyisoflavone

Fig. 6 Basic structure of the flavanones, flavones and isoflavones.

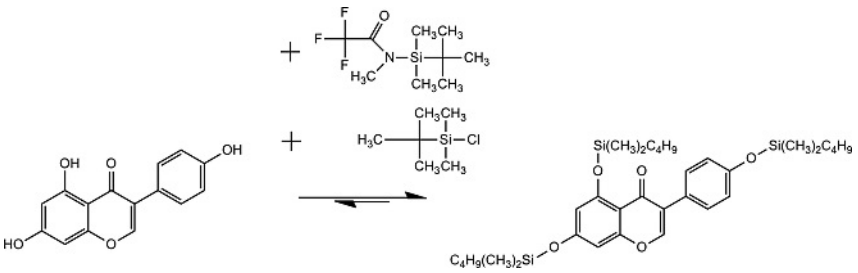


Fig. 7 Derivatisation reaction using genistein as example.