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ORIGINAL PAPER

Simultaneous monitoring of seven phenolic metabolites of endocrine disrupting compounds (EDC) in human urine using gas chromatography with tandem mass spectrometry

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Abstract A gas chromatographic-tandem mass spectrometric (GC-MS/MS) method for the simultaneous determination of the three well-known endocrine disruptors, bisphenol A, daidzein and genistein, as well as of four human pesticide metabolites which are supposed to have proper endocrine activity or which are metabolites of endocrine-disrupting compounds, viz., 1- and 2-naphthol, 2-isopropoxyphenol and 3,5,6-trichloropyridinol, has been developed and validated. The method involves enzymatic cleavage of the conjugates using β-glucuronidase/arylsulfatase followed by solid-phase extraction and derivatisation with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide. Isotopically labelled internal standards were used for all analytes, to achieve best analytical error correction. The method proved to be both sensitive and reliable in human urine with detection limits ranging from 0.1 to 0.6 μg/L for all analytes. Precision and repeatability was determined to range from 1 to 15 %. Compared with other published analytical procedures, the present method enables the simultaneous determination of a couple of phenolic agents with competitive or improved analytical reliability. Thus, the present method is suitable for a combined monitoring of the exposure to prominent xenobiotics with effects on the human endocrine system (bisphenol A, carbaryl, chlorpyrifos, chlorpyrifos-methyl, naphthalene, propoxur, triclopyr) and phytoestrogens (daidzein, genistein) in population studies.

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Introduction

The analysis of human biological samples for exogenous substances, which are known or assumed to modify the human endocrine system, is of great interest for population studies dealing with these health effects. Substances of phenolic structure are an important group of xenobiotics effective on the human endocrine system [1]. An important representative of this group of substances is bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA), for which effects on the endocrine system and its development have been demonstrated in vitro as well as in vivo [2–4]. BPA is mainly applied as a key component for the production of epoxy resins and polycarbonate plastic and therefore it is a component in several consumer goods and commodities. Due to its widespread use, it has become a ubiquitously occurring contaminant. After absorption into the human body, BPA is excreted via urine almost entirely conjugated to glucuronic acid [5, 6]. Further, human exposures to phenolic substances with endocrine activity may result from pesticides and their residues in the diet [7]. Pesticides the metabolism of which results in an exposure to phenolic substances are chlorpyrifos, chlorpyrifos-methyl, triclopyr, propoxur, carbaryl and naphthalene (Fig. 1). Chlorpyrifos and chlorpyrifos-methyl are organophosphate pesticides, for which endocrine effects were demonstrated [8]. Both pesticides were degraded to 3,5,6-trichloropyridinol (2,3,5-trichloro-6-hydroxypyridine, TCPy), which is eliminated via urine. Furthermore, TCPy is derived by the metabolism of the herbicide trichlopyr, also [9–11] (Fig. 1).

Another phenolic compound of concern is 2-isopropoxyphenol (2-(1-methylethoxy)-phenol, IPP), which

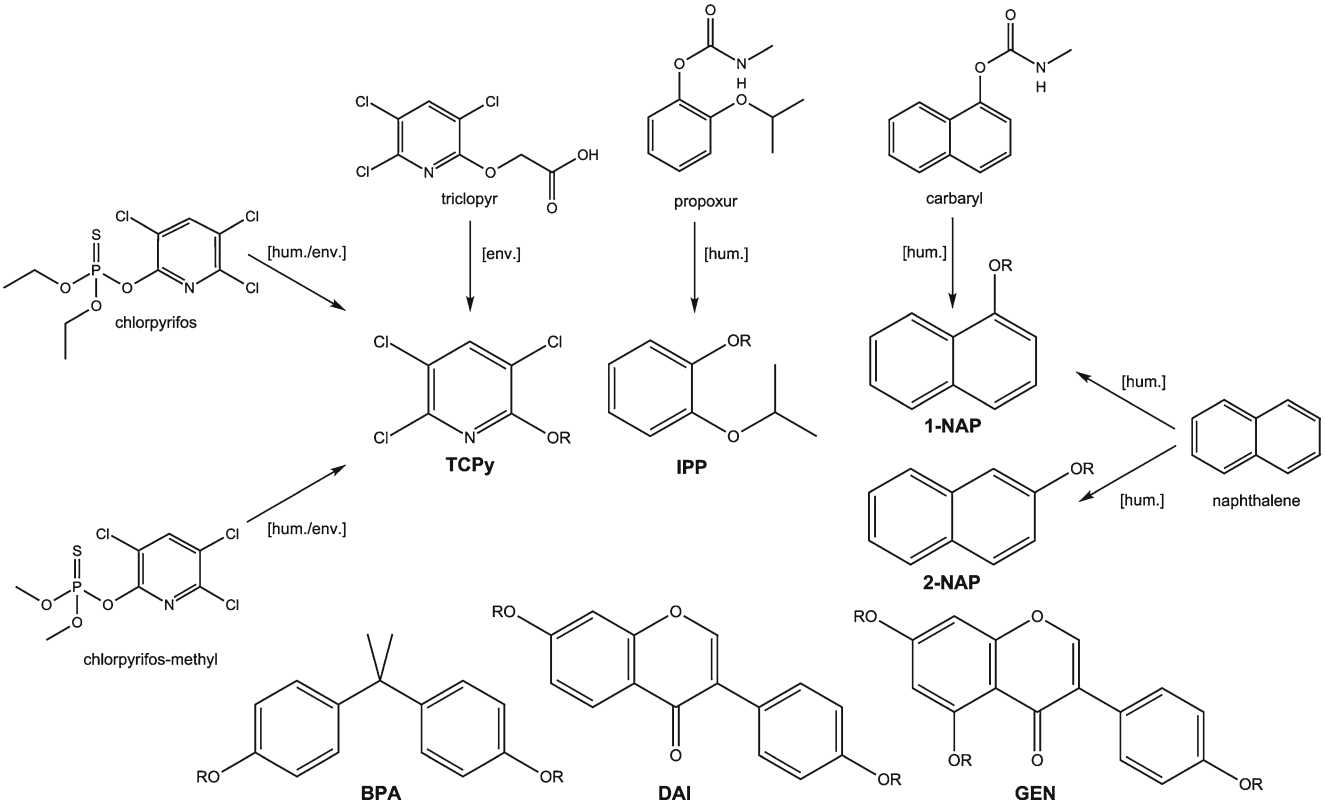

Fig. 1 Chemical structure of the analytes (R= H) and their analytical derivatives (R= $SiMe_2tBu$) including their corresponding precursor compounds. 3,5,6-trichloropyridinol (TCPy), 2-isopropoxyphenol (IPP), 1-naphthol (1-NAP), 2-naphthol (2-NAP), bisphenol A (BPA), daidzein (DAI) and genistein (GEN); human metabolism (hum.), environmental degradation (env.)

is a human metabolite of propoxur. The concentration of IPP in urine has been shown to be a well-suited biomarker of a latent exposure to propoxur [12–14] (Fig. 1). Propoxur is a carbamate insecticide, which is used in many households, mainly for non-professional pest control. In cell assays with human breast and endometrial cancer cells, it exhibits weak capacity to displace radiolabelled oestrogen or progesterone from oestrogen or progesterone receptor, which reveals its ability for endocrine effects [15].

1-Naphthol (1-hydroxynaphthalene, 1-NAP) is a urinary human metabolite of both, the widely used insecticide carbaryl and of naphthalene. 2-Naphthol (2-hydroxynaphthalene, 2-NAP) is a second important metabolite of naphthalene, but not of carbaryl (Fig. 1). Thus, the combined monitoring of both naphthols also allows to trace the parent compounds in case of an unknown exposure situation [16]. 1- and 2-NAP are suspected to act as endocrine-disruptive compounds (EDCs), especially via thyroid hormone receptor antagonist activity [17]. Exposures to 1-NAP were associated with diminished serum testosterone levels and a loss in human semen quality [18].

Besides these mentioned xenobiotics, people are additionally exposed to many naturally occurring endocrine disruptors such as the phytoestrogens genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, GEN) and daidzein (7-hydroxy-3-(4-hydroxy phenyl)-4H-1-benzo pyran-4-one, DAI) which may be extensively ingested via diet. After absorption in the human body, GEN and DAI are eliminated via urine in conjugate forms mainly [19]. GEN and DAI (Fig. 1) are isoflavones, which are particularly contained in soy products and are often concerned with beneficial health effects such as blood cholesterol lowering [20], vascular protection [21] and osteoporosis prevention [22]. In contrast, there are a few contradictory studies, investigating possible adverse impact of isoflavone intake on human fertility and reproductive parameters, such as sperm motility and morphology [23–26], probably induced via endocrine activity. Therefore, several studies were performed dealing with the possible isoflavone hazard [25, 27–29].

In epidemiological studies, the common questions are, whether adverse effects are resulted from synergistic or additive effects of several compounds and whether a major agent can be identified in a group of suspected modulators [4, 30, 31]. The application of a multi-compound method for the simultaneous determination of a broad range of EDC biomarkers gives a crucial advantage to achieve these objectives. The comparable physical

and chemical properties of EDC biomarkers with the same chemical structure support the application of a single analytical procedure for the simultaneous determination of these parameters. Since all the mentioned EDC metabolites (BPA, 1-/2-NAP, IPP, TCPy, as well as GEN and DAI) basically contain phenolic structures (Fig. 1), it is obvious to create and apply a unified method for their determination. Thus, we developed a simple, reliable and highly sensitive method for the simultaneous monitoring of these EDC biomarkers in the urine of individuals of the general population, using gas chromatography coupled with tandem mass spectrometry (GC-MS/MS).

Experimental

Chemicals and materials

BPA, DAI, GEN, IPP, 1-NAP, 2-NAP, TCPy and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide in combination with 1 % *tert*-butyldimethylchlorosilane (MtBSTFA) were purchased from Sigma Aldrich (Steinheim, Germany) each at highest purity available (>98 %). The internal standard substances 2,2′,6,6′-$^{2}H_4$-bisphenol A (BPA-D4, purity 98 %), 1-O^{2}H-2,3,4,5,6,7,8-$^{2}H_7$-naphthalene (1-NAP-D8, purity 98 %) and 2-OH-1,3,4,5,6,7,8-$^{2}H_7$-naphthalene (2-NAP-D7, purity 98 %) were obtained from CDN Isotopes Inc. (Quebec, Canada). DAI-D3 (3′,5′,8-$^{2}H_3$-daidzein, purity 97 %) solution (60 μg/mL in acetonitrile) and GEN-D4 (3′,5′,6,8-$^{2}H_4$-genistein, purity 94 %) solution (100 μg/mL in acetonitrile) were supplied by Cambridge Isotope Laboratories (Andover, USA). TCPy-^{13}C3 (2,3,4-$^{13}C_3$-2,3,5-trichloro-6-hydroxypyridine, purity >95 %) and IPP-D4 (3,4,5,6-$^{2}H_4$-2-isopropoxyphenol, purity 98 %) were custom synthesised by the Institute for Organic and Biomolecular Chemistry (Göttingen, Germany). Acetonitrile (MeCN, dry, >99 %), methanol (MeOH, dry, GC grade), toluene (dry, GC grade), acetic acid (glacial) and sodium hydroxide (p.a.) were purchased from Merck (Darmstadt, Germany). β-glucuronidase/arylsulfatase from *Helix pomatia* was supplied by Roche Diagnostics GmbH (Mannheim, Germany).

Polystyrene-divinylbenzene copolymer columns for solid-phase extraction (SPE) (Isolute-101, 25 mg sorbent, 1-mL capacity, average particle size 65 μm (irregular-shaped particles), pore diameter 100 Å) were obtained from Biotage AB (Uppsala, Sweden).

Standard preparation

The calibration material was split into three groups (group 1: DAI and GEN; group 2: 1-NAP and 2-NAP; group 3: BPA, TCPy and IPP) by preparing working solutions with different concentration levels for each group, because of different expected ranges. Stock solutions of BPA, DAI, GEN, 1-NAP, 2-NAP, TCPy and IPP were prepared by dissolving the standard substances in MeCN. Working solutions I and II were prepared by dilution of the stock solutions with water. Working solution I contained 5 mg/L of 1-NAP and 2-NAP; working solution II contained 5 mg/L DAI and GEN, respectively. Concentration levels of 400 μg/L each of BPA, TCPy and IPP were contained in working solution III. The stock solutions of the internal standards BPA-D4, DAI-D3, GEN-D4, 1-NAP-D8, 2-NAP-D7, TCPy-^{13}C3 and IPP-D4 were prepared by dissolving the standard substances in MeCN and then were diluted with water to obtain a working solution of the internal standards (5 mg/L each of 1-NAP-D8, 2-NAP-D7, DAI-D3 and GEN-D4, 500 μg/L each of BPA-D4, TCPy-^{13}C3 and IPP-D4).

Calibration procedure

The calibration was carried out using seven calibration levels prepared by spiking of pooled urine with different volumes of working solutions I, II and III to achieve final concentrations of 2–250 μg/L each of DAI and GEN, 1–100 μg/L each of 1-NAP and 2-NAP and 0.4–16 μg/L each of BPA, TCPy and IPP. Additionally, identical pooled urine was used as a blank sample and was included in each analytical series. The calibration standards were processed as described in "Sample preparation (standard procedure)". Linear calibration curves were obtained by plotting the quotients of the analytes' peak areas to the peak areas of the corresponding labelled internal standards as a function of spiked concentration.

Sample preparation (standard procedure)

To eliminate sample contamination with environmentally occurring free BPA, the used glassware was heated to 180 °C for a minimum of 2 h prior to the sample preparation procedure. Aliquots of urine samples were stored frozen at −18 °C until analysis. Initially, urine samples were thawed, equilibrated to room temperature and vortex-mixed. For hydrolysis, an aliquot of 1 mL urine was transferred into a glass vial containing 50 μL of the internal standard working solution and 500 μL of sodium acetate hydrolysis buffer (400 mmol/L, pH=5.0) to adjust the pH of the sample to the enzymes optimum value. Subsequently, 10 μL of β-glucuronidase/arylsulfatase was added, briefly vortex-mixed, and then the sample was incubated for 3 h at 37 °C. To assess possible sample contamination with ubiquitously free BPA or with DAI and GEN from contaminated enzyme solution, every sample preparation series contained a blank sample, consisting of all used reagents and purified water instead of urine. After the hydrolysis step, the sample was passed through an Isolute-101 SPE column which was preconditioned successively with 500 μL MeOH, 250 μL MeCN and

twice with 500 μL sodium acetate buffer (without vacuum). The column was then washed successively with 500 μL hydrolysis buffer, 750 μL water and 500 μL MeOH (50 % *v/v*). Afterwards, the column was first dried by centrifugation for 10 min at 1,900×*g* and then air-dried by applying vacuum for 3 min. The elution was carried out with 800 μL MeCN, directly into a 2-mL glass vial containing 200 μL of toluene. Subsequently, the eluate was concentrated under a gentle stream of nitrogen to a volume of 200 μL and was passed on to the derivatisation process.

Derivatisation process (standard procedure)

The derivatisation was performed by addition of 30 μL MtBSTFA. Then the sealed vials were incubated at room temperature for a minimum of 30 min. After derivatisation, the samples were concentrated to a total volume of 100 μL by evaporation under a nitrogen stream and subsequently analysed by GC-MS/MS.

Optimisation of sample preparation and derivatisation

The sample preparation process was optimised with regard to the proportion of MeOH in the aqueous washing solution, used for SPE. Therefore, identical pooled urine samples spiked with 8 μg/L BPA, TCPy and IPP, 50 μg/L 1- and 2-NAP and 100 μg/L DAI and GEN, respectively, were transferred to the SPE columns and rinsed with water containing increasing amounts of 0 to 90 % MeOH. Mean absolute peak areas were plotted against the methanol content in the washing solution to reveal the degree of analyte loss during clean-up (Fig. 2).

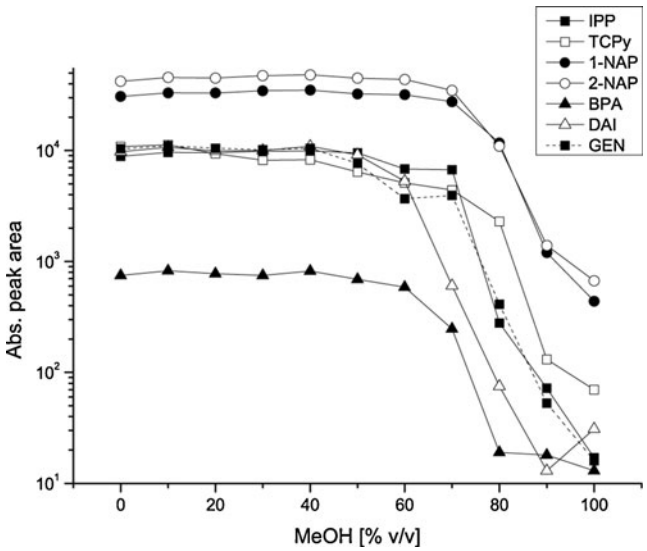

Fig. 2 Mean absolute peak areas of IPP, TCPy, 1-NAP, 2-NAP, BPA, DAI, and GEN in relation to the used percentage of MeOH in the washing solution during SPE (n=2)

The applicability of MtBSTFA for derivatisation of the studied compounds was initially checked by analysis of reaction mixtures containing 1 mg/L of each of the analytes in MeCN/toluene (1:1) and a surplus of MtBSTFA. These reaction mixtures were allowed to react at room temperature for 24 h and subsequently were analysed by GC-MS in full scan mode. For further optimization of the derivatisation procedure, identical reaction mixtures containing 1 mg/L of each of the analytes were determined after varying incubation conditions, i.e., two different temperatures (25 and 60 °C) and incubation times ranging from 10 to 120 min. To study the influence of the MtBSTFA quantity used for derivatisation, identical reaction mixtures with different proportions of derivatisation reagent ranging from 5 to 50 % MtBSTFA were analysed. Mean absolute peak area values were plotted against time of derivatization (Fig. 3) and against concentration of MtBSTFA to analyse the optimal derivatization conditions.

Gas chromatography–tandem mass spectrometry (GC-MS/MS)

GC-MS/MS was performed on an Agilent 7000A Series Triple Quadrupole GC/MS, consisting of an Agilent 7890A gas chromatograph equipped with a split/splitless injector, containing a deactivated single taper helix liner, an Agilent AS7693 autosampler and the Agilent 7000A Series triple quadrupole GC-MS EI/CI mainframe, with EI ion source installed (Agilent Technologies, Santa Clara, USA). A sample volume of 1 μL was injected into the inlet assembly in splitless mode with purge flow to vent after 1.2 min and an injector temperature of 320 °C. For gas chromatographic separation, a 5 %-phenyl-arylene-95 %-dimethylpolysiloxane low-bleed capillary column (ZB-5 ms 30 m×250 μm×0.25 μm+5 m retention gap) (Phenomenex, Aschaffenburg, Germany) was used at a constant flow of 1.2 mL/min helium carrier gas. The initial column temperature of 120 °C was held for 2 min, then raised at a rate of 20 °C/min to 240 °C and subsequently raised at a rate of 50 °C/min to 320 °C, remaining at this level for 8 min. The temperature of the transfer line was set to 320 °C. The ion source was operating at −70 eV, at a temperature of 230 °C and with a solvent delay of 5.5 min. The mass selective detector was adjusted to 150 °C quadrupole temperature and the collision gas (nitrogen) used for collision-induced dissociation (CID) was set to a flow rate of 1.5 mL/min. For detection, the mass spectrometer was working in the multiple reaction monitoring mode (MRM). To establish the MS/MS operating conditions, standard solutions of each analyte and internal standard substance were determined separately. Two mass transitions of the most sensitive precursor ions were optimised regarding product ions and corresponding CID energy for every analyte. The mass

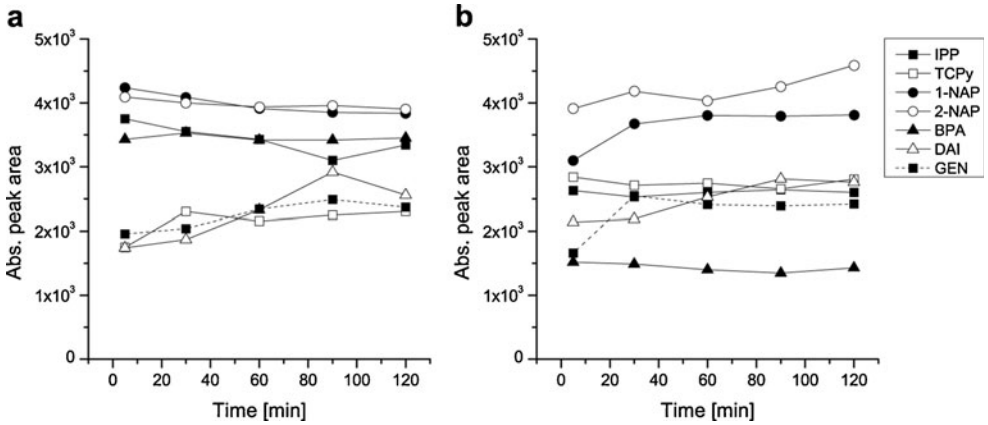

Fig. 3 Mean absolute peak areas (n=2) of IPP, TCPy, 1-NAP, 2-NAP, BPA, DAI, and GEN in relation to the reaction time during derivatisation at **a** 25 °C and **b** 60 °C

transition with the higher intensity was used as quantifier, the second as qualifier. GC retention times and optimised MRM parameters, thus obtained for every single analyte and internal standard substance, are summarised in Table 1.

Quality control

Quality control materials were prepared by spiking pooled urine samples with two different concentration levels of all analytes. For a low concentration quality control material (Q_{low}), pooled urine was spiked with 2 µg/L BPA, TCPy and IPP; 15 µg/L 1- and 2-NAP and 25 µg/L DAI and GEN, respectively. For preparation of a high-concentration quality-control material (Q_{high}), pooled urine was spiked with 8 µg/L each of BPA, TCPy and IPP; 50 µg/L each of 1- and 2-NAP and 100 µg/L each of DAI and GEN. The quality control material was divided into aliquots of 1 mL and stored at −18 °C. One Q_{low} and one Q_{high} sample have been analysed during each analytical series.

Validation

The precision and repeatability of the method was determined using intra- and inter-day relative standard deviations. Precision was determined by analysing pooled urine spiked with two concentration levels (Q_{low} and Q_{high} material), each ten times in a row. By analysing one Q_{low} and one Q_{high}-sample on three different days, repeatability was determined.

Accuracy was calculated as relative recovery of the analyte concentration in the spiked samples (Q_{low} and Q_{high}-material). Absolute recovery rate, which particularly embraces the losses during solid-phase extraction, was determined as quotient of mean absolute peak area counts of three

Table 1 Retention times and MRM-specific parameters of the analytes and internal standard substances

Analyte	t_R [min]	Quantifier ion [m/z]		CID [V]	Qualifier ion [m/z]		CID [V]
		Precursor ion (Q1)	Product ion (Q3)		Precursor ion (Q1)	Product ion (Q3)	
IPP	6.13	167	151	10	167	136	30
IPP-D4	6.13	171	155	10	171	140	30
TCPy	7.00	254	219	10	256	221	10
TCPy-^{13}C3	7.08	259	224	10	261	226	10
1-NAP	7.80	201	185	15	201	145	17
1-NAP-D8	7.78	208	192	15	208	152	17
2-NAP	7.90	201	145	17	201	185	15
2-NAP-D7	7.89	208	152	17	208	192	15
BPA	10.80	456	441	12	456	207	12
BPA-D4	10.78	460	445	12	460	209	12
DAI	14.60	425	397	24	425	283	37
DAI-D3	14.59	428	400	24	428	286	37
GEN	16.27	555	539	48	555	483	42
GEN-D4	16.25	559	543	48	559	487	42

t_R retention time, *CID* collision-induced dissociation, $Q1$ quadrupole 1, $Q3$ quadrupole 3

separate urine samples, spiked with each analyte before and after solid-phase extraction.

Limits of detection (LOD) and limits of quantification (LOQ) were determined by means of a seven equidistant point calibration in pooled urine, according to guideline DIN 32 645 [32]. Additionally, the LODs were calculated using a peak-to-peak height signal to noise ratio of 3:1, at the lowest calibration concentration of each analyte (cf. Calibration procedure).

Contamination control and error correction

To identify possible contaminations with ubiquitously free BPA, a blank value which undergoes the whole preparation process and contains all reagents and water instead of urine was simultaneously analysed in every measurement series. The BPA content of this sample should be kept as low as possible or ideally be less than the LOQ. If there are measurement series containing blank samples showing BPA contents within the range of LOQ and LOQ+3s (s= 0.1 μg/L; absolute Q_{low} precision of BPA), i.e., 0.3–0.6 μg/L, the acquired BPA concentration is included in the calculation. Series showing higher BPA blank content were rejected and analysed for a second time.

Results and discussion

Sample preparation and derivatisation

Due to the diversity of physical properties of the target analytes, the challenge was to design a sample preparation procedure, which enables maximum selectivity and clean-up for all analytes. A strong non-polar, hydrophobic polystyrene–divinylbenzene copolymer phase (Isolute® 101) specially designed for the extraction of polar chemicals, which are not sufficiently retained by C8 or C18 material, was selected for this purpose. As a result, we found quantitative recovery rates for almost all analytes (Table 2). Moreover, we discovered a constant retention of all analytes on the SPE phase, even during clean-up using wash solutions with high shares of methanol. The mean absolute peak areas of all investigated compounds show only slight variations even if the loaded SPE column is washed with up to 50 % aqueous MeOH (Fig. 2). Consequently, the observed high affinity of the analytes to the polymeric phase enables a thorough clean-up of the urine samples during preparation, by rinsing of weakly bound polar organic matrix compounds with aqueous MeOH.

Regarding derivatisation, pure MtBSTFA has previously been used as derivatisation reagent for TCPy [33] and BPA [34]. In combination with 1 % tBDMCS, it also proved to be a well-suited reagent for the reaction with isoflavones [35]. In recent analytical studies, IPP was mainly derivatized using 2,3,4,5,6-pentafluorobenzyl bromide (PFBBr) [12] because PFBBr is a commonly used derivatisation reagent for most phenolic compounds. Further experiences exist for the derivatization of IPP with 1-chloro-3-iodopropane [36]. Moreover, the mass spectrometric behaviour of trimethylsilylated 2-alkoxyphenols has been extensively investigated [37]. Hence, MtBSTFA was supposed to react with IPP as well. This was confirmed by the analysis of reaction mixtures consisting of reference substances and MtBSTFA. The experiments showed that MtBSTFA reacts with all free hydroxy functions of the analytes. Further, optimization showed that the derivatisation process of all analytes at room temperature is almost independent from reaction time. However, higher variations were observed when derivatisation took place at 60 °C (Fig. 3). Additionally, our experiments showed that for almost all analytes, maximum absolute area counts were achieved when the reaction mixture contained at least 50 % of MtBSTFA (Fig. 4). Therefore, the final optimised derivatisation process was performed at 25 °C for 30 min with 50 % MtBSTFA in the reaction mixture.

Table 2 Validation data in urine

Analyte	LOD[a] [μg/L]	LOQ[a] [μg/L]	Q_{low} [μg/L]	Q_{high} [μg/L]	Precision [%]		Repeatability [%]		Accuracy [%] (n=3)	Abs. recovery [%] (n=3)
					Q_{low} (n=3)	Q_{high} (n=3)	Q_{low} (n=3)	Q_{high} (n=3)		
IPP	0.5	1.7	2.1	8.5	6.3	1.2	11.6	2.3	101–103	97
TCPy	0.2	0.7	2.0	8.1	2.1	1.7	4.3	1.5	100–103	111
1-NAP	0.2	0.9	13.0	46.7	6.1	0.4	6.2	1.5	92–103	90
2-NAP	0.1	0.4	17.6	63.5	2.7	1.4	8.3	0.3	96–103	96
BPA	0.1	0.3	2.0	8.0	5.0	2.0	6.0	5.1	91–105	104
DAI	0.3	1.1	25.1	100.3	2.0	1.0	5.1	2.0	95–99	40
GEN	0.6	2.0	24.9	99.4	2.9	3.8	14.5	2.2	85–109	60

[a] LOD and LOQ calculated according to DIN 32645 [32]; LOD values calculated as 3 $s0$, with peak-to-peak height signal-to-noise ratio ($s0$): IPP= 0.2 μg/L, TCP=0.07 μg/L, 1-NAP=0.03 μg/L, 2-NAP=0.02 μg/L, BPA=0.01 μg/L, DAI=0.06 μg/L, GEN=0.08 μg/L

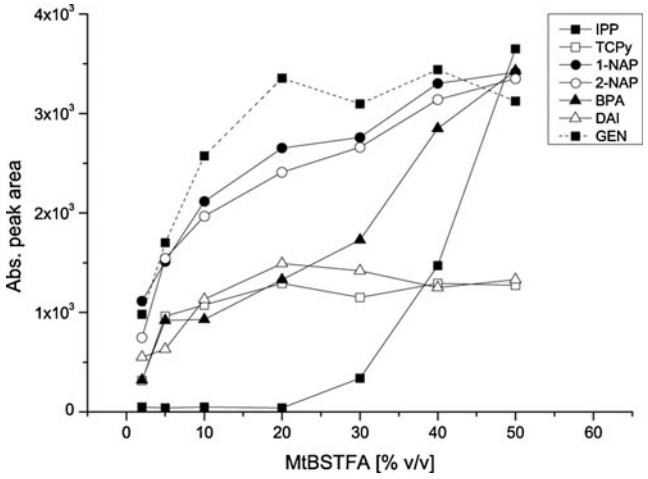

Fig. 4 Mean absolute peak areas of IPP, TCPy, 1-NAP, 2-NAP, BPA, DAI, and GEN (1 mg/L each) in relation to the used percentage of MtBSTFA+1 % tBDMCS during derivatisation (n=2)

Due to the sample preparation by solid-phase extraction, a contamination with ubiquitously occurring free BPA is potentially possible [38]. Taking precautions, such as thermic pre-preparation of all glassware and the use of fresh reagents, resulted in sufficiently clean blank samples. By the application of the procedure in a set of 15 series, a mean blank of BPA of 0.4±0.2 µg/L was determined.

GC-MS/MS conditions

Because of the wide span of molecular weights of the derivatized compounds, which ranges from 258 g/mol for the naphthol derivatives to 612 g/mol for the GEN derivative, the simultaneous determination of these substances was a challenging task. A steep temperature gradient was elaborated, mainly to prevent peak broadening of the isoflavone signals and to reduce analysis time. As a result, all analytes show baseline separation within an optimum runtime of only 16.5 min (Fig. 5).

The *tert*-butyldimethylsilyl derivatives of the analytes and the internal standards were registered each by a specific precursor ion, which undergoes collision-induced dissociation (CID) forming at least two product ions, one used as quantifier, the other as qualifier ion.

In accordance with Krauss et al. [37], IPP forms a stable cyclic precursor ion of m/z 167, $[C_8H_{11}O_2Si]^+$ during electron ionisation (EI), by loss of a *tert*-butyl radical from the silyl group and a stable *iso*-propyl radical from the *ortho* position. This precursor ion is then dissociated into two-product ions, by successive loss of methyl radicals from the silyl group (see Electronic Supplementary Material (ESM) Fig. S1). Because of the formation of the stable cyclic precursor ion of IPP, it is absolutely necessary to use an isotope-labelled internal standard for IPP, which is deuterated at the benzene ring position, but not at the isopropyl substituent.

The precursor ion $[C_7H_7Cl_3NOSi\ ]^{\bullet+}$ of the *tert*-butyldimethylsilyl derivative of TCPy shows a characteristic natural isotopic ion distribution of chlorine with m/z 254 (100 %) and m/z 257 (13 %), after the loss of a *tert*-butyl radical from the silyl group, due to electron ionisation. The precursor ion $[C_4{}^{13}C_3H_7Cl_3NOSi]^{\bullet+}$ of the internal standard TCPy-^{13}C3 shows a similar spectrum, which is increased by 3 Da with m/z 257 (100.0 %), 259 (99.3 %). Since the internal standard is labelled only three times with ^{13}C, the spectra of TCPy and TCPy-^{13}C3 significantly overlap at m/z 257, with TCPy contributing to about 13 % of the ions counted (see ESM Fig. S2). This is the reason why the less affected ion m/z 259 and not the labelled counterpart to m/z 254 i.e. m/z 257 is used for quantification. Another approach to solve this problem would possibly be the use of a higher labelled internal standard. However, the crucial point is that the addition of the internal standard does not influence the quantity of the analyte ions because TCPy-^{13}C3 is not containing any isotopes with m/z 254 or 256.

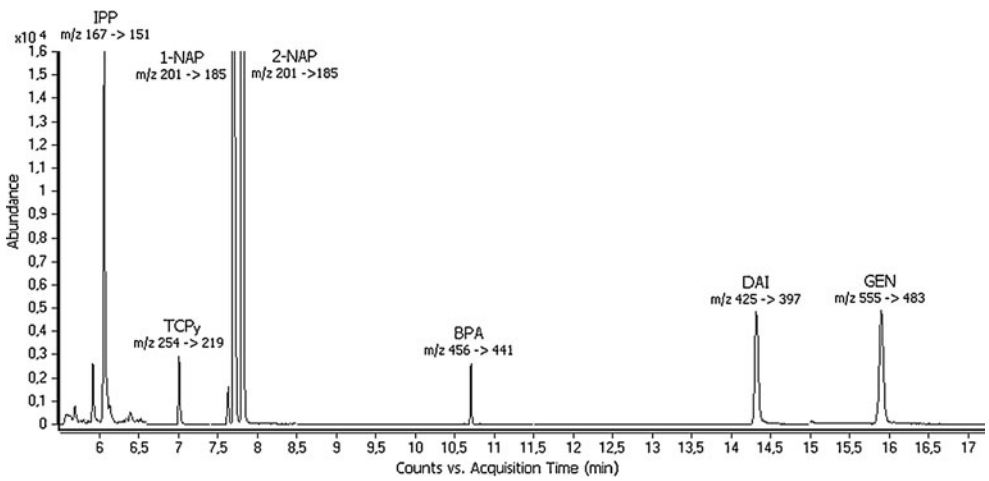

Fig. 5 GC-MS/MS MRM chromatogram of a calibration standard in urine containing 11 µg/L each of IPP, TCPy and BPA, 70 µg/L each of 1- and 2-NAP and 200 µg/L each of DAI and GEN

The TCPy derivative shows only one stable mass transition during CID at all collision energies ranging from 1 to 40 V, which is the secession of one chlorine radical. Therefore, the two isotopically different precursor ions *m/z* 254 and 256 have been used as qualifier and quantifier ions, instead of one precursor ion which is split off into two product ions (Table 1).

Regarding the mass spectrometric data of 1- and 2-NAP, both analytes show the same precursor ion and are equally dissociated into the same two product ions. The only difference is, that the product ions show different intensities depending on the analyte. Therefore, qualifier and quantifier ion are reversed for 1-NAP and 2-NAP (Table 1). As 1-NAP-D8 contains a deuterated hydroxyl group, it loses one deuterium during the derivatisation procedure. Hence, both internal naphthol standards show the same precursor ion and mass transitions.

The *tert*-butyldimethylsilyl derivative of BPA produces a stable precursor molecular ion $[M]^{\bullet+}$ *m/z* 456, during EI, that undergoes fragmentation to the qualifier ion $[M\text{-}15]^{\bullet+}$ *m/z* 441and the qualifier ion $[M\text{-}234]^{\bullet+}$ *m/z* 207, which is formed through C–C cleavage of the methyl bridge. The quantifier ion *m/z* 441 was also previously described using GC-MS in selected ion monitoring mode [35]. However, the use of tandem mass spectrometry leads to higher selectivity, which is essential for avoiding false positive results.

Reliability of the method

Linearity of the calibration graphs was given for all analytes for the observed calibration ranges: 0.4–16.0 μg/L each for IPP, TCPy and BPA, 1.0–100.0 μg/L each for 1- and 2-NAP and 2.0–250.0 μg/L each for DAI and GEN. The coefficients of correlation of the calibration curves were higher than r=0.995 for all analytes. Intraday precision was determined by analysis of Q_{low} and Q_{high}-material in a row and ranged from 2.0 to 6.3 % (Q_{low}) and 1.0 to 3.8 % (Q_{high}), respectively, for all analytes. Slightly higher results were generally found for interday repeatability, which ranged from 4.3 to 14.5 % (Q_{low}) and 0.3 to 5.1 % (Q_{high}), respectively (Table 2). Appropriate isotopically labelled internal standard substances were used for all analytes for error correction during sample workup. Therefore, optimum accuracy values around 100 % were achieved for all analytes (Table 2). Nevertheless, the good performance of the SPE procedure is demonstrated by satisfying absolute recovery rates, which mainly represent low losses during SPE, for almost all analytes (Table 2). Only the isoflavone fraction shows decreased but still sufficient recovery rates. Comparing accuracy and recovery rates, the use of labelled internal standards proved to be very efficient for compensation of such errors.

The LOD levels in the urine matrix were found to range between 0.1 and 0.6 μg/L. Hence, the corresponding LOQ levels range from 0.3 to 2.0 μg/L. Thereby, GEN shows the highest LOQ of 2.0 μg/L within the set of analytes (Table 2). For better comparability to other published methods, the LODs were additionally calculated using a signal to noise ratio of 3:1. The LODs calculated this way were remarkably lower but even more imprecise, than the LODs calculated according to DIN (Table 2).

During the last two decades, several procedures were published for the determination of EDC containing phenolic structures (Table 3). However, the majority of these procedures were developed and validated for the determination of a single biomonitoring parameter only. Hardt and Angerer [12] presented a procedure for the determination of IPP in urine, using derivatization with PFBBr and GC-single quadrupole MS. The procedure enables a limit of detection of 0.5 μg/L, which is the same as the present method. Bartels et al. [33] and Koch and Angerer [39] provided analytical procedures for the determination of TCPy in urine, using MtBSTFA derivatization and for specific detection GC-MS/MS and GC-single quadrupole MS, respectively. Whereas Bartels and co-workers stated a LOD of 0.5 μg/L, Koch and Angerer estimated a LOD of 0.05 μg/L, based on the threefold signal-to-noise ratio. The reason for the lower LOD of the second method may be due to the application of steam distillation for the clean-up in this procedure. However, steam distillation is not applicable to every phenolic analyte as well as implies a prolonged preparation procedure. Many methods were developed for the determination of bisphenol A in urine in the last decade, which were presented and discussed in the review of Dekant and Völkel [5]. Most of these procedures are using GC-MS and LC-MS techniques. Three of them are listed in Table 3, which are representative for the collection of BPA biomonitoring methods. Völkel et al. [6] and Ye et al. [40] applied the LC-MS/MS technique for the determination of BPA, without any derivatization. The LOD of these procedures were stated to be 0.3 and 0.4 μg/L, respectively, which are higher than the LOD of the present method. Ye and co-worker combined the LC-MS/MS detection with an online-SPE that resulted in very simple and comfortable procedure. Moreover, they were able to determine six further environmental phenols in human urine, but none of the additional parameters of the present method. Arakawa et al. [41] applied a procedure for the determination of BPA using *N*,*O*-bis(trimethylsilyl) trifluoro acetamide (BSTFA) for derivatization and GC-MS/MS technique, which enables a LOD of 0.38 μg/l. Grace et al. [42] developed a procedure for the determination of several isoflavones in human urine using BSTFA derivatization and GC-MS. Their procedure enables the determination of daidzein and genistein with LOD of 1.3 μg/L, which is higher than for the present method. Campo et al. [43] developed an analytical procedure for the determination of monohydroxy metabolites of polycyclic aromatic compounds using BSTFA derivatization and GC-MS technique.

Table 3 Comparison of the present method with other published methods dealing with the analysis of the respective analytes

Author	LOD (LOQ) [μg/L]							LOD/LOQ calculated	Method
	IPP	TCPy	1-NAP	2-NAP	BPA	DAI	GEN		
Present method	0.5 (1.7)	0.2 (0.7)	0.2 (0.9)	0.1 (0.4)	0.1 (0.3)	0.3 (1.1)	0.6 (2.0)	a	GC/MS-MS (MRM)
Hardt et al. 1999 [12]	0.5	–	–	–	–	–	–	b	GC/MS (SIM)
Koch and Angerer 2001 [39]	–	0.05 (0.1)	–	–	–	–	–	b (c)	GC/MS (SIM)
Bartels et al. 1992 [33]	–	0.5	–	–	–	–	–	b	GC/MS-MS (MRM)
Bravo et al. 2005 [36]	0.4	0.4	0.3	0.2	–	–	–	b	GC/MS-MS (MRM)
Yoshida and Yoshida 2012 [44]	–	(2.4)	(3.8)	(3.7)	–	–	–	(g)	GC/MS (SIM)
Campo et al. 2008 [43]	–	–	(0.9)	(0.9)	–	–	–	f	GC/MS (SIM)
Grace et al. 2003 [42]	–	–	–	–	–	1.3	1.3	e	GC/MS (SIM)
Moors et al. 2007 [35]	–	–	–	–	3 (7)	4 (9)	5 (18)	a	GC/MS (SIM)
Arakawa et al. 2004 [41]	–	–	–	–	0.38	–	–	b	GC/MS-MS (MRM)
Ye et al. 2005 [40]	–	–	–	–	0.4	–	–	b	LC/MS-MS (MRM)
Völkel et al. 2008 [6]	–	–	–	–	0.3 (1.25)	–	–	b (d)	LC/MS-MS (MRM)

a LOD and LOQ calculated according to DIN 32645 [32] calibration function method in urine; *b* LOD=3*s0*; *c* LOQ=6*s0*; *d* LOQ=13*s0*; *e* LOD=3 *SDq* + *q*; *f* LOQ=(3 *SDq* + *q*)/*m*; with signal-to-noise ratio (*s0*) and standard deviation of the intercept (*SDq*), slope (*m*), and intercept (*q*) of a calibration function in urine; *g* LOQ calculated as ten times standard deviation at the lowest point of the calibration curve

For the analytes 1-NAP and 2-NAP, they found a LOQ of 0.9 μg/L, which is the same as the present method for 1-NAP and about twice as high as the present method for 2-NAP. Moors et al. [35] combined the determination of the isoflavones daidzein and genistein with the determination of BPA using SPE, MBSTF derivatization and GC-MS detection. The LODs of their procedure are 3 μg/L for BPA, 4 μg/l for DAI and 5 μg/l for GEN, which are distinctly higher than for the present method. Another conjunctive method was provided by Yoshida and Yoshida [44], which enables the determination of 3,5,6-trichloro-2-pyridinol, 1- and 2-naphthol beside other phenols and several phosphates. The procedure bases on solid-phase extraction, derivatization with MtBSTFA and detection by GC-MS. The authors stated LOQ for TCPy of 2.4 μg/L, 3.8 μg/L for 1-NAP and 3.7 μg/L for 2-NAP, which were also distinctly higher than for the present method. Bravo et al. [36] published a procedure for the determination of several environmental phenols in human urine which includes the analysis of IPP, TCPy, 1- and 2-NAP. They applied a solid-phase extraction, a derivatization with 1-chloro-3-iodopropane to generate chloropropyl ether derivatives of the analyte and GC-tandem mass spectrometry. The LODs of the procedure for IPP (0.4 μg/L), TCPy (0.4 μg/L), 1-NAP (0.3 μg/L) and 2-NAP (0.2 μg/L) were similar or slightly higher than the present method.

However, the comparability of LODs and LOQs is affected by the different approaches which were applied for their estimation by the different authors. In most publications, LODs and LOQs were calculated using signal-to-noise ratios [6, 12, 33, 36, 39], whereas the limits of the present method were calculated based on an equidistant-calibration function close to the LOD.

Conclusion

The present method is the first procedure, which enables the simultaneous determination of the seven endocrine agents BPA, DAI, GEN, IPP, 1-NAP, 2-NAP and TCPy in human urine. The use of a polystyrene–divinylbenzene copolymer for solid-phase extraction facilitates the enrichment and the separation of the analytes from the matrix with only small losses with the exception of DAI and GEN. However, the higher losses of DAI and GEN during the procedure are no crucial drawbacks due to their high urinary levels in the general population and due to the use of isotope-labelled internal standards, which enables a total compensation of the losses. The optimised silylation procedure ensures a rapid and complete derivatisation of all phenolic analytes. Accordingly, the validation data demonstrates a high reproducibility, accuracy and sensitivity of the present method, which may be derived

from the use of isotope-labelled internal standards for each parameter, particularly. Compared with other published analytical procedures for the determination of the phenolic substances, the present method enables the simultaneous determination of a broad spectrum of biomarkers, with competitive or improved analytical sensitivity. Thus, the present method is recommendable for a combined monitoring of the exposure to prominent xenobiotics with endocrine activity (bisphenol A, carbaryl, chlorpyrifos, chlorpyrifos-methyl, naphthalene, propoxur, triclopyr) and phytoestrogens (daidzein, genistein), in population studies.

References

1. Hu JY, Aizawa T (2003) Quantitative structure-activity relationships for estrogen receptor binding affinity of phenolic chemicals. Water Res 37(6):1213–1222
2. National Toxicology Programme, Center for the Evaluation of Risks to Human Reproduction, U.S. Department of Health and Human Services Food and Drug Administration F (2008) Monograph on the potential human reproductive and developmental effect of bisphenol A. NIH Publication No. 08-5994
3. European Food Safety Authority, EFSA Panel (2007) Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to 2,2-bis(4-hydroxyphenyl)propane (bisphenol A). EFSA J 428:1–75. doi:10.2903/j.efsa.2007.428
4. Kortenkamp A (2007) Ten years of mixing cocktails: a review of combination effects of endocrine-disrupting chemicals. Environ Health Perspect 115:98–105. doi:10.1289/ehp.9357
5. Dekant W, Völkel W (2008) Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures. Toxicol Appl Pharmacol 228(1):114–134
6. Völkel W, Kiranoglu M, Fromme H (2008) Determination of free and total bisphenol A in human urine to assess daily uptake as a basis for a valid risk assessment. Toxicol Lett 179(3):155–162. doi:10.1016/j.toxlet.2008.05.002
7. Ejaz S, Akram W, Lim CW, Lee JJ, Hussain I (2004) Endocrine disrupting pesticides: a leading cause of cancer among rural people in Pakistan. Exp Oncol 26(2):98–105
8. Gore AC (2001) Environmental toxicant effects on neuroendocrine function. Endocrine 14(2):235–246
9. Racke KD (1993) Environmental fate of chlorpyrifos. Rev Environ Contam Toxicol 131:1
10. Nolan R, Rick D, Freshour N, Saunders J (1984) Chlorpyrifos: pharmacokinetics in human volunteers. Toxicol Appl Pharmacol 73(1):8–15
11. Carmichael N, Nolan R, Perkins J, Davies R, Warrington S (1989) Oral and dermal pharmacokinetics of triclopyr in human volunteers. Hum Exp Toxicol 8(6):431
12. Hardt J, Angerer J (1999) Gas chromatographic method with mass-selective detection for the determination of 2-isopropoxyphenol in human urine. J Chromatogr B 723(1–2):139–145
13. Leenheers LH, van Breugel DC, Ravensberg JC, Meuling WJA, Jongen MJM (1992) Determination of 2-isopropoxyphenol in urine by capillary gas chromatography and mass-selective detection. J Chromatogr B 578(2):189–194. doi:10.1016/0378-4347(92)80415-m
14. Krechniak J, Foss W (1979) Determination of propoxur and 2-isopropoxyphenol in blood, urine and tissues. Bull Environ Contam Toxicol 23(1):531–535. doi:10.1007/bf01769999
15. Klotz DM, Arnold SF, McLachlan JA (1997) Inhibition of 17 beta-estradiol and progesterone activity in human breast and endometrial cancer cells by carbamate insecticides. Life Sci 60(17):1467–1475. doi:10.1016/s0024-3205(97)00098-2
16. Meeker JD, Barr DB, Serdar B, Rappaport SM, Hauser R (2006) Utility of urinary 1-naphthol and 2-naphthol levels to assess environmental carbaryl and naphthalene exposure in an epidemiology study. J Expo Sci Environ Epidemiol 17(4):314–320
17. Sun H, Shen OX, Xu XL, Song L, Wang XR (2008) Carbaryl, 1-naphthol and 2-naphthol inhibit the beta-1 thyroid hormone receptor-mediated transcription in vitro. Toxicology 249(2–3):238–242. doi:10.1016/j.tox.2008.05.008
18. Meeker JD, Ryan L, Barr DB, Herrick RF, Bennett DH, Bravo R, Hauser R (2004) The relationship of urinary metabolites of carbaryl/naphthalene and chlorpyrifos with human semen quality. Environ Health Perspect 112(17):1665–1670. doi:10.1289/ehp.7234
19. Clarke DB, Lloyd AS, Botting NP, Oldfield MF, Needs PW, Wiseman H (2002) Measurement of intact sulfate and glucuronide phytoestrogen conjugates in human urine using isotope dilution liquid chromatography-tandem mass spectrometry with [13C3] isoflavone internal standards. Anal Biochem 309(1):158–172
20. Taku K, Umegaki K, Ishimi Y, Watanabe S (2008) Effects of extracted soy isoflavones alone on blood total and LDL cholesterol: meta-analysis of randomized controlled trials. Ther Clin Risk Manag 4(5):1097–1103
21. Siow RCM, Mann GE (2010) Dietary isoflavones and vascular protection: activation of cellular antioxidant defenses by SERMs or hormesis? Mol Aspects Med 31(6):468–477
22. Taku K, Melby MK, Nishi N, Omori T, Kurzer MS (2011) Soy isoflavones for osteoporosis: an evidence-based approach. Maturitas 70(4):333–338. doi:10.1016/j.maturitas.2011.09.001
23. Fraser LR, Beyret E, Milligan SR, Adeoya-Osiguwa SA (2006) Effects of estrogenic xenobiotics on human and mouse spermatozoa. Hum Reprod 21(5):1184–1193. doi:10.1093/humrep/dei486
24. Chavarro JE, Toth TL, Sadio SM, Hauser R (2008) Soy food and isoflavone intake in relation to semen quality parameters among men from an infertility clinic. Hum Reprod 23(11):2584–2590. doi:10.1093/humrep/den243
25. Mitchell JH, Cawood E, Kinniburgh D, Provan A, Collins AR, Irvine DS (2001) Effect of a phytoestrogen food supplement on reproductive health in normal males. Clin Sci 100(6):613–618
26. Song G, Kochman L, Andolina E, Herko RC, Brewer KJ, Lewis V (2006) Beneficial effects of dietary intake of plant phytoestrogens on semen parameters and sperm DNA integrity in infertile men. Fertil Steril 86(3):S49
27. Messina M (2011) Evidence does not support the conclusion that soy is an endocrine disruptor. J Pediatr Endocrinol Metab 24(9–10):859–860
28. Bar-El Dadon S, Reifen R (2010) Soy as an endocrine disruptor: cause for caution? J Pediatr Endocrinol Metab 23(9):855–861
29. Andres S, Abraham K, Appel KE, Lampen A (2011) Risks and benefits of dietary isoflavones for cancer. Crit Rev Toxicol 41(6):463–506
30. Gray L, Wilson VS, Stoker T, Lambright C, Furr J, Noriega N, Howdeshell K, Ankley GT, Guillette L (2006) Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals1. Int J Androl 29(1):96–104. doi:10.1111/j.1365-2605.2005.00636.x
31. Kortenkamp A (2008) Low dose mixture effects of endocrine disrupters: implications for risk assessment and epidemiology. Int J Androl 31(2):233–240. doi:10.1111/j.1365-2605.2007.00862.x
32. DIN 32645 (2008) Chemical analysis—decision limit, detection limit and determination limit under repeatability conditions—terms, methods, evaluation. Beuth, Berlin
33. Bartels MJ, Kastl PE (1992) Analysis of 3,5,6-trichloropyridinol in human urine using negative-ion chemical ionization gas chromatography–mass spectrometry. J Chromatogr B Biomed Sci Appl 575(1):69–74. doi:10.1016/0378-4347(92)80505-k

34. Xu J, Wu L, Chen W, Chang AC (2008) Simultaneous determination of pharmaceuticals, endocrine disrupting compounds and hormone in soils by gas chromatography–mass spectrometry. J Chromatogr A 1202(2):189–195
35. Moors S, Blaszkewicz M, Bolt HM, Degen GH (2007) 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(7):787–798
36. Bravo R, Caltabiano LM, Fernandez C, Smith KD, Gallegos M, Whitehead RD Jr, Weerasekera G, Restrepo P, Bishop AM, Perez JJ, Needham LL, Barr DB (2005) Quantification of phenolic metabolites of environmental chemicals in human urine using gas chromatography–tandem mass spectrometry and isotope dilution quantification. J Chromatogr B 820(2):229–236. doi:10.1016/j.jchromb.2005.03.012
37. Krauss D, Mainx HG, Tauscher B, Bischof P (1985) Fragmentation of trimethylsilyl derivatives of 2-alkoxyphenols: a further violation of the 'even-electron rule'. Org Mass Spectrom 20(10):614–618. doi:10.1002/oms.1210201005
38. Koch HM, Kolossa-Gehring M, Schröter-Kermani C, Angerer J, Brüning T (2012) Bisphenol A in 24 h urine and plasma samples of the German Environmental Specimen Bank from 1995 to 2009: a retrospective exposure evaluation. J Expo Sci Environ Epidemiol 22:610–616. doi:10.1038/jes.2012.39
39. Koch HM, Angerer J (2001) Analysis of 3,5,6-trichloro-2-pyridinol in urine samples from the general population using gas chromatography–mass spectrometry after steam distillation and solid-phase extraction. J Chromatogr B Biomed Sci Appl 759(1):43–49. doi:10.1016/s0378-4347(01)00209-2
40. Ye X, Kuklenyik Z, Needham LL, Calafat AM (2005) Automated on-line column-switching HPLC-MS/MS method with peak focusing for the determination of nine environmental phenols in urine. Anal Chem 77(16):5407–5413
41. Arakawa C, Fujimaki K, Yoshinaga J, Imai H, Serizawa S, Shiraishi H (2004) Daily urinary excretion of bisphenol A. Environ Health Prev Med 9(1):22–26
42. Grace PB, Taylor JI, Botting NP, Fryatt T, Oldfield MF, Bingham SA (2003) Quantification of isoflavones and lignans in urine using gas chromatography/mass spectrometry. Anal Biochem 315(1):114–121
43. Campo L, Rossella F, Fustinoni S (2008) Development of a gas chromatography/mass spectrometry method to quantify several urinary monohydroxy metabolites of polycyclic aromatic hydrocarbons in occupationally exposed subjects. J Chromatogr B 875 (2):531–540
44. Yoshida T, Yoshida J (2012) Simultaneous analytical method for urinary metabolites of organophosphorus compounds and moth repellents in general population. J Chromatogr B 880:66–73