Talanta 129 (2014) 209–218

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

A multiclass method for the analysis of endocrine disrupting chemicals in human urine samples. Sample treatment by dispersive liquid–liquid microextraction

F. Vela-Soria, O. Ballesteros *, A. Zafra-Gómez, L. Ballesteros, A. Navalón *

Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, University of Granada, Campus of Fuentenueva, E-18071 Granada, Spain

ARTICLE INFO

Article history:
Received 17 January 2014
Received in revised form
6 May 2014
Accepted 12 May 2014
Available online 29 May 2014

Keywords:
Endocrine disrupting chemicals
Human urine analysis
Dispersive liquid–liquid microextraction
GC–MS/MS

ABSTRACT

The population is continuously exposed to endocrine disrupting chemicals (EDCs). This has influenced an increase in diseases and syndromes that are more frequent nowadays. Therefore, it is necessary to develop new analytical procedures to evaluate the exposure with the ultimate objective of establishing, in an accurate way, relationships between EDCs and harmful health effects. In the present work, a new method based on a sample treatment by dispersive liquid–liquid microextraction (DLLME) for the extraction of six parabens (methyl-, ethyl-, isopropyl-, propyl-, isobutyl and butylparaben), six benzophenones (benzophenone-1, benzophenone-2, benzophenone-3, benzophenone-6, benzophenone-8 and 4-hydroxybenzophenone) and two bisphenols (bisphenol A and bisphenol S) in human urine samples, followed by gas chromatography-tandem mass spectrometry (GC–MS/MS) analysis is proposed. An enzymatic treatment allows determining the total content of the target EDCs. The extraction parameters were accurately optimized using multivariate optimization strategies. Ethylparaben ring-$^{13}C_6$ and bisphenol A-d_{16} were used as surrogates. Found limits of quantification ranging from 0.2 to 0.5 ng mL^{-1} and inter-day variability (evaluated as relative standard deviation) ranging from 2.0% to 14.9%. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery rates ranged from 94% to 105%. A good linearity, for concentrations up to 300 ng mL^{-1} for parabens and 40 ng mL^{-1} for benzophenones and bisphenols, respectively, was obtained. The method was satisfactorily applied for the determination of target compounds in human urine samples from 20 randomly selected individuals.

1. Introduction

In the last century, as a consequence of the huge industrial development, wildlife and humans are exposed to synthetic chemicals that can interfere with the normal functioning of the endocrine system. These compounds, commonly called endocrine disrupting chemicals (EDCs), are present in many types of products, such as personal care products (PCPs), pharmaceuticals, sunscreens, foodstuffs, beverage cans, *etc.* Nowadays, a large amount of research groups are working to demonstrate the relationship between human exposure to EDCs and some diseases such as hypospadias, cryptorchidism, testicular cancer, loss in semen quality, breast cancer, many uterine and ovarian diseases and many anomalies in the age of puberty [1–6]. These evidences imply greater efforts to assess human exposure to EDCs.

There are a lot of families of compounds that are able to induce an endocrine disrupting response in the human organism. In the present study, bisphenol A (BPA) and bisphenol S (BPS), parabens (PBs) and benzophenones (BPs), have been selected as the target EDCs, due to their widespread presence in very common products and stuffs that are continuously consumed by population.

BPA is one of the most representative compounds of the EDCs group. It is a highly reactive compound used as the raw material in a large amount of manufactured products, such as polycarbonate plastics, epoxy resins used to line metal cans, and in many plastic consumer products including toys, water pipes, drinking containers, eyeglass lenses, sports safety equipment, dental monomers, medical equipment and tubing and consumer electronics [7]. BPA is one of the highest volume chemicals produced worldwide, with an estimated production of 5.5 million tons [8]. Although humans are frequently exposed to BPA through multiple sources, the diet is considered as the major source of exposure [9].

Another important family of EDCs is parabens. Because of their low toxicity and cost, their inert nature and worldwide regulatory

* Corresponding author. Tel.: +34 958 243326; fax: +34 958 243328.
E-mail address: oballest@ugr.es (O. Ballesteros).

http://dx.doi.org/10.1016/j.talanta.2014.05.016
0039-9140/

acceptance, parabens are widely used in PCPs, pharmaceuticals and food or beverages [10,11]. Individually or in combination, parabens are used in over 13,200 formulations in nearly all types of cosmetics, being the most commonly used methyl and propylparaben [11]. Exposure to parabens may occur through inhalation, dermal contact or ingestion [10,12].

The third group of EDCs considered in this work is benzophenones. There are 12 well-described BPs, namely benzophenone-1 (BP-1) to benzophenone-12 (BP-12), as well as other less usual compounds as 2-hydroxybenzophenone (2-OH-BP) or 4-hydroxybenzophenone (4-OH-BP). In cosmetics and PCPs, BP-1 and BP-3 are usually used in the formulation of nail polishes and enamels. These BPs are also used in the manufacturing of bath, makeup, hair or skin care products and sunscreens [13]. These compounds protect cosmetics and PCPs from damage by absorbing, reflecting, or scattering UV rays. When they are used as sunscreen ingredients, BP-3 and BP-4 protect the skin from UV rays. Human exposures to BPs can be through the skin and ingest, being the most important the dermal route [14].

Disrupting abilities of BPA, PBs and BPs have been demonstrated in many *in vitro* and *in vivo* studies. These compounds are able to induce the proliferation of MCF-7 cancerous cells, demonstrating a clear estrogenic character [15–19]. Animal exposure modeling has proved that these chemicals produce an abnormal sexual development, erratic behaviors and carcinogenesis in adult animals [20–22], and the offspring of exposed individuals can also suffer endocrine disorders during fetal and early post-natal development, therefore permanent adverse effects can be caused [23,24]. In fact, this phenomenon could be present in human population, as it is suggested in several epidemiologic studies where it is shown a negative correlation between prenatal EDCs exposure and measures of cognitive skills in childhood [6,25,26].

Although there are some differences about EDCs biotransformation depending on exposure via and specific chemical structure characteristics, animal and human organisms are able to transform BPA, PBs and BPS in β-D-glucuronide derivatives easily excreted through the urine because of their high water solubility [14,27–33]. However, free forms of EDCs can accumulate in certain human tissues due to their lipophilic nature producing harmful disrupting effects and passing to the offspring. Several analytical studies have demonstrated that these chemicals are present in human placental tissue [34–36], as well as in human milk [37–39].

Due to the complexity of biological matrices, new methods for sample treatment are needed to ensure good results in exposure analysis. These new procedures have to be simple and fast, and provide enough sensitivity to detect very low quantities of EDCs. The use of the highly-potential microextraction techniques as the dispersive liquid–liquid microextraction (DLLME), developed by Rezaee and co-workers in 2006 [40], have provided good results in complex samples. The fundament of the DLLME has been explained elsewhere, as well as the advantages over the traditional extraction techniques and other microextraction techniques [40]. DLLME has been widely used in the analysis of many types of pollutants and organic compounds in environmental matrices, in food samples and in biological human samples [41,42]. However, DLLME has hardly been used in analysis of EDCs in human samples. Recently, some methods for the determination of BPs and PBs in human urine and serum samples by DLLME–LC–MS/MS or DLLME–LC–DAD have been proposed [43–46].

The aim of the present work is to develop a selective and sensitive DLLME followed by the GC–MS/MS analytical method for the simultaneous determination of six parabens, six benzophenones and two bisphenols in human urine samples. The proposed method was validated and satisfactorily applied to determine the EDCs content (free and total) in samples collected from 20 unknown volunteers.

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). Methylparaben (MP), ethylparaben (EP), isopropylparaben (IsPP), propylparaben (PP), isobutylparaben (IsBP) and butylparaben (BP) were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA), bisphenol S (BPS), labeled deuterium bisphenol A (BPA-d_{16}), benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP) and ethylparaben ring $^{13}C_6$ labeled (EP-$^{13}C_6$) were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions (100 mg L^{-1}) for each compound were prepared in methanol and stored at 4 °C in the dark. These solutions were stable for at least four months. Working standards were prepared just before use, diluted with methanol.

The 4-methylumbelliferyl glucuronide, 4-methylumbelliferyl sulfate and β-glucuronidase/sulfatase (*Helix pomatia*, H1) were purchased from Sigma-Aldrich. $^{13}C_4$-4-methylumbelliferone was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). A mixture of $^{13}C_4$-4-methylumbelliferone, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl glucuronide was prepared in water and stored at 4 °C until use. The enzyme was prepared daily by dissolving 10 mg of β-glucuronidase/sulfatase (3×10^6 U g solid^{-1}) in 1.5 mL of 1 M ammonium acetate/acetic acid buffer solution (pH 5.0).

Methanol, ethanol, acetone, ethyl acetate and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Sodium chloride and ammonium acetate were supplied by Panreac (Barcelona, Spain). Chlorobenzene (ClBz), tricloromethane (TCM), carbon tetrachloride (TCC), potassium chloride, creatinine, sodium sulfate, hippuric acid, ammonium chloride, citric acid, magnesium sulfate, sodium phosphate monobasic monohydrate, calcium chloride dihydrate, oxalic acid, lactic acid, glucose, sodium metasilicate nonahydrate, pepsin and N,O-Bis(trimethylsilyl)trifluoro-acetamide with trimethylchlorosilane (BSTFA/1% TMCS) were purchased from Sigma-Aldrich.

2.2. Instrumentation and software

GC–MS/MS analysis was performed using an Agilent 7890 GC (Agilent Technologies, Palo Alto, *CA*, USA) equipped with a split-splitless inlet and a 7693 ALS autosampler. The detector was an Agilent 7000B triple quadrupole mass spectrometer with inert electron-impact ion source. The mass spectrometer worked in the SRM mode. Electron impact (EI) ionization at −70 eV was used. Agilent MassHunter B.03.02 software package was used for control and data analysis. Helium (99.9999% purity) was used as carrier gas and quench gas (a gas employed in the Agilent 7000 mass spectrometer), and nitrogen (99.999% purity) was used as collision gas; both gases were supplied by Air Liquide España S.L. (Madrid, Spain).

All pH measurements were carried out with a Crison (Crison Instruments S.A., Barcelona, Spain) combined glass-Ag/AgCl (KCl 3 M) electrode using a previously calibrated Crison 2000 digital pH-meter. A MS-100 thermo shaker (Optimum Ivymen System, Cornecta, Spain) was used to make the enzymatic treatment.

Statgraphics Plus version 5.0 software package (Manugistics Inc., Rockville, MD, USA, 2000) was used for statistical and regression analyses (linear mode).

2.3. Sample collection and storage

Human urine samples were collected from 20 volunteers (10 male and 10 female). Samples were anonymized, frozen at −86 °C

and stored until analysis in our laboratory. All volunteers signed their informed consent to participate in the study.

2.4. Sample treatment

2.4.1. Enzymatic treatment

In order to evaluate free and total amounts of EDCs in urine, each sample was treated in two different ways. One sample was processed without addition of enzymes and the other one was treated with β-glucuronidase/sulfatase. For experiments without enzymatic treatment, an aliquot of urine (5.0 mL) was added into a centrifuge glass tube and spiked with 50 μL of surrogates (EP-$^{13}C_6$ and BPA-d_{16}) standard solution (5 mg L^{-1} of EP-$^{13}C_6$, 2 mg L^{-1} of BPA-d_{16}). To analyze the total (free+conjugated) concentration of the tested EDCs, 5.0 mL of sample was spiked with 50 μL of surrogates (EP-$^{13}C_6$ and BPA-d_{16}) standard solution and 100 μL of enzyme solution (β-glucuronidase/sulfatase). Furthermore, 125 μL of 4-methylumbelliferyl glucuronide/4-methylumbelliferyl sulfate/$^{13}C_4$-4-methylumbelliferone standard mixture (4 μg mL^{-1}) were added to check the extent of the deconjugation. After mixing, the sample was incubated at 37 °C for 24 h. 4-methylumbelliferyl sulfate and 4-methylumbelliferyl glucuronide were deconjugated to free 4-methylumbelliferone. The 4-methylumbelliferone/$^{13}C_4$-4-methylumbelliferone peak area ratio was monitored to assess the correct action of the enzyme. Deconjugation efficiencies were close to 100% in all cases.

2.4.2. DLLME procedure

Prior to DLLME procedure the aliquot of human urine (5 mL) was diluted to 10.0 mL with 10% NaCl aqueous solution (w/v). The pH was adjusted to 2.0 with 0.1 M HCl. This solution was placed in a 15 mL screw-cap glass test tube. Next, 0.5 mL of acetone (disperser solvent) and 750 μL of TCM (extraction solvent) were mixed and injected rapidly into the aqueous sample with a syringe. The mixture was gently shaken for 10 s, and centrifuged for 20 min at 4000 rpm (2600xg). All sedimented phase volume was transferred to a clean glass vial using a 1.0 mL micropipette. The organic phase was evaporated under a nitrogen stream. The residue was dissolved with 100 μL of a mixture of ethyl acetate and BSTFA/1% TMCS (80:20; v/v). After mixing, the solution was heated to 60 °C for 20 min. At this point, the extract was ready to be analyzed.

2.5. Gas chromatography-mass apectrometric analysis

Analytes were separated on a HP–5MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) from Agilent. The injection port of the GC was set at 250 °C. Samples were automatically injected using the splitless-injection mode. The injection volume was 1 μL. The helium carrier gas (99.999% purity) flow was maintained at 1 mL min^{-1}. The initial oven temperature was set at 70 °C (held for 2.0 min). Then, three linear ramps were established in order to reach 120 °C at 25 °C min^{-1}, 260 °C at 10 °C min^{-1} (this temperature was hold for 2 min) and finally 280 °C at 20 °C min^{-1}. This final temperature was maintained for 5 min. Total time of analysis was 26 min. The SRM method was created in the triple quadrupole mass spectrometer. Two ions were monitored for each analyte, the first for quantification and the second one for confirmation. Table 1 shows the selected mass spectrometer conditions.

The method was divided into 3 segments to obtain enough sampling points for each chromatographic peak and adequate dwell times to obtain good sensitivity. Resolution was adjusted to 1.0 Da for quadrupole 1 and 3. Temperatures of the transfer line, ion source and quadrupole 1 and 2 were 290 °C, 300 °C and 150 °C respectively. Mass spectrometer auto-tune was performed on a weekly basis.

3. Results and discussion

3.1. Optimization of DLLME conditions

3.1.1. Selection of extraction and disperser solvents

The disperser-extractant solvent pair is one of the most important factors in DLLME optimization. In the first step, aliquots of 5.0 mL of spiked human urine at a concentration of 10 ng mL^{-1} of all studied EDCs were treated as is described above. Mixtures of 1.0 mL of different disperser solvents (acetonitrile, methanol, ethanol and acetone) and 0.1 mL of extractant solvent (TCC, TCM and ClBz) were injected to 10 mL of sample solution (three experimental replicates). The highest responses for all compounds corresponded to acetone-TCM pair. Some examples that support

Table 1
Selected SRM transitions and optimized potentials.

Compound	Transitions	CE (eV)	t_R (min)	Compound	Transitions	CE (eV)	t_R (min)
MP	224.0→209.1[a] 224.0→131.1[b]	5 30	8.9	**BP-3**	285.1→242.1[a] 285.1→212.0[b]	20 30	15.4
EP	244.0→229.2[a] 244.0→201.1[b]	5 10	9.7	**BP-1**	343.2→271.1[a] 343.2→105.1[b]	20 30	16.0
EP-$^{13}C_6$	244.0→292.2[a] 244.0→201.1[b]	10 10	9.7	**BPA-d_{16}**	386.0→368.3[a] 368.0→197.1[b]	10 30	16.5
IsPP	252.0→195.1[a] 252.0→210.1[b]	15 10	10.1	**BPA**	372.0→191.2[a] 357.0→191.2[b]	20 30	16.6
PP	252.0→195.1[a] 252.0→210.1[b]	15 10	10.8	**BP-8**	373.2→73.0[a] 299.1→73.0[b]	30 30	16.9
IsBP	210.0→195.1[a] 210.0→151.1[b]	5 15	11.4	**BP-6**	403.0→360.1[a] 403.0→73.0[b]	20 30	18.8
BP	210.0→195.1[a] 210.0→151.1[b]	5 15	12.0	**BP-2**	519.3→447.1[a] 519.1→147.0[b]	30 30	20.1
4-OH-BP	270.0→193.1[a] 270.0→255.1[b]	10 10	14.9	**BPS**	394.0→379.1[a] 394.0→213.0[b]	10 10	20.9

CE, Collision energy; tR, Retention time.

[a] SRM transition used for quantification.
[b] SRM transition for confirmation.

Table 2
Experimental domain and standardized effects of investigated factors.

Factor	Level	
	Low	High
Acetone volume (mL)	0.5	3.5
TCM volume (μL)	150	750
Sample pH	2	6
NaCl (%)	0	10
Shaking time (s)	10	120

Factor	Standarized effect values													
	MP	EP	IsPP	PP	IsBP	BP	BPA	BPS	4-OH-BP	BP-1	BP-2	BP-3	BP-6	BP-8
Acetone volume (A)	0.210[a]	0.737[a]	0.044[a]	0.176[a]	0.064[a]	0.182[a]	0.210[a]	-0.116[a]	0.139[a]	0.113[a]	0.112[a]	0.082[a]	0.089[a]	0.104[a]
TCM volume (B)	0.627[a]	1.214[a]	0.206[a]	0.428[a]	0.260[a]	0.770[a]	0.761[a]	0.019	0.338[a]	0.424[a]	0.294[a]	0.273[a]	0.270[a]	0.374[a]
pH (C)	−0.066[a]	−0.861[a]	−0.086[a]	−0.280[a]	−0.123[a]	−0.369[a]	−0.371[a]	−0.241[a]	−0.142[a]	−0.199[a]	−0.114[a]	−0.126[a]	−0.137[a]	−0.181[a]
% NaCl (D)	0.007	0.287[a]	0.003	−0.006	−0.003	−0.040	−0.028	0.203[a]	−0.004	−0.019	0.026	−0.009	0.010	−0.015
Shaking Time (E)	−0.157[a]	−0.105[a]	−0.059[a]	−0.083[a]	−0.069[a]	−0.203[a]	−0.217[a]	−0.177[a]	−0.113[a]	−0.110[a]	−0.055[a]	−0.065[a]	−0.091[a]	−0.099[a]
AB	0.263[a]	0.881[a]	0.046[a]	0.154[a]	0.060[a]	0.181[a]	0.189[a]	0.131[a]	0.141[a]	0.105[a]	0.093[a]	0.067[a]	0.078[a]	0.092[a]
AC	0.107[a]	−0.578[a]	0.050[a]	−0.021	0.054[a]	0.154	0.140[a]	0.122[a]	−0.027	0.085[a]	0.014	0.043[a]	0.024	0.067[a]
AD	−0.104[a]	−0.074[a]	−0.040[a]	−0.067[a]	−0.047[a]	−0.176[a]	−0.141[a]	−0.152[a]	−0.045[a]	−0.087[a]	−0.011	−0.053[a]	−0.014	−0.064[a]
AE	−0.091[a]	−0.258[a]	0.009	0.008	0.004	0.0336	−0.003	0.167[a]	−0.040	0.007	0.006	−0.009	−0.024	−0.001
BC	−0.084[a]	−0.949[a]	−0.041[a]	−0.207[a]	−0.082[a]	−0.254[a]	−0.205[a]	0.088[a]	−0.105[a]	−0.109[a]	−0.091[a]	−0.063[a]	−0.087[a]	−0.101[a]
BD	−0.096[a]	−0.005	−0.021	−0.022	−0.021	−0.081	−0.081	−0.131[a]	−0.014	−0.049	0.007	−0.027	0.004	−0.034[a]
BE	−0.165[a]	−0.244[a]	0.005	0.001	0.006	0.017	0.031	0.103[a]	−0.026	0.018	−0.016	0.015	−0.014	0.014
CD	−0.041[a]	−0.040[a]	0.009	−0.014	0.005	0.027	0.027	−0.140[a]	−0.038	0.013	0.008	0.015	−0.001	0.019
CE	−0.027[a]	0.210[a]	−0.019	−0.024	−0.014	−0.070	−0.070	0.110[a]	−0.031	−0.050	−0.001	−0.031	0.014	−0.028
DE	0.171[a]	−0.298[a]	0.043[a]	−0.028	0.039[a]	0.108[a]	0.133[a]	−0.139[a]	−0.008	0.081[a]	0.013[a]	0.052[a]	0.031	0.067[a]

[a] Statistically significant factors (95% confidence level).

this conclusion are shown as supplementary material (Figure S01). Since pairs including methanol or ethanol did not offer clean extracts, they were discarded in all cases.

3.1.2. Effects of the volumes of extractant and dispersant, pH sample, salt addition and extraction time

The effects of sample pH, salt (NaCl) percentage, extraction time (defined as the period during which the sample is shaken after addition of the binary extraction mixture and before centrifugation), volume of extractant and volume of dispersant on the performance of the method were simultaneously investigated using a two-level 2^{5-1} fractional factorial design, with three replicates of the central point. In order to minimize the content of EDCs that are naturally found in human urine samples, a pool with very low concentration of these compounds was spiked at a concentration of 10 ng mL^{-1} of all EDCs and used in diagnostic and optimization studies. The residue was dissolved with 100 μL of a mixture of ethyl acetate and BSTFA/1% TMCS (80:20; *v*/*v*) containing 10 ng mL^{-1} of EP-$^{13}C_6$ and 10 ng mL^{-1} of BPA-d_{16}. The response variable used for these experiments was relative area. Experimental domain and standardized effects of factors are summarized in Table 2.

All variables have a significant influence (95% confidence level) to at least one analyte in the extraction procedure. The influence of pH, NaCl content and extraction time was the same for all compounds and the higher responses were obtained at low pH values, high percentage of NaCl and low extraction time. Therefore, in order to simplify the optimization process, a pH value of 2, 5% (*w*/*v*) of NaCl and 10 s of shaking time were selected as optimal. The samples were diluted 1:1 (*v*/*v*) by using a 10% NaCl solution to finally obtain a final percentage of 5% NaCl and the pH was adjusted to 2.0 with 0.1 M HCl.

3.1.3. Volume of extractant and dispersant

The optimal volume of acetone and TCM were evaluated with the Doehlert surface response design (three central point replicates). This experimental design allows the simultaneous

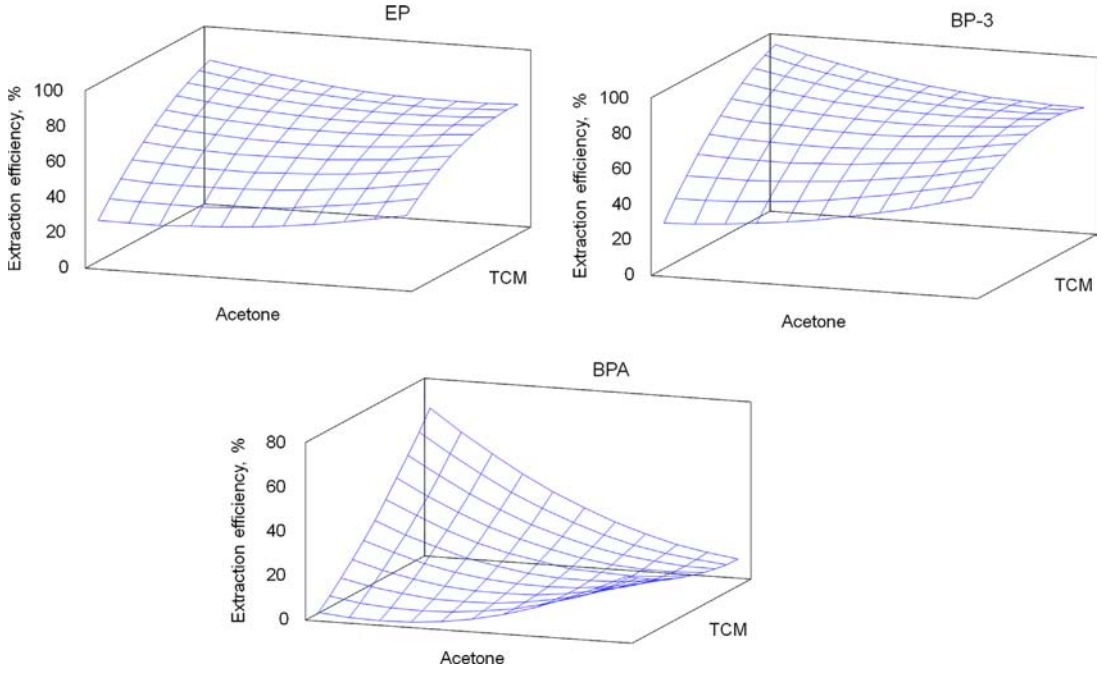

Fig. 1. Response surfaces obtained by the Doehlert design.

Table 3
Analytical and statistical parameters.

	a	s_a	*b* (mL ng^{-1})	s_b (mL ng^{-1})	R^2 (%)	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	LDR (ng mL^{-1})
MP	-6.2×10^{-2}	3.1×10^{-4}	3.2×10^{-2}	2.3×10^{-4}	99.8	0.06	0.2	0.2–300
EP	-2.9×10^{-2}	1.6×10^{-4}	1.7×10^{-2}	1.1×10^{-4}	99.8	0.08	0.3	0.3–300
IsPP	-2.1×10^{-1}	1.4×10^{-3}	1.1×10^{-1}	5.5×10^{-4}	99.9	0.04	0.2	0.2–300
PP	-4.1×10^{-1}	2.1×10^{-3}	2.1×10^{-1}	1.5×10^{-3}	99.8	0.08	0.3	0.3–300
IsBP	-5.9×10^{-1}	3.1×10^{-3}	2.9×10^{-1}	1.5×10^{-3}	99.9	0.1	0.4	0.4–300
BP	6.6×10^{-1}	3.7×10^{-3}	3.2×10^{-1}	2.6×10^{-3}	99.8	0.1	0.4	0.4–300
BPA	-8.9×10^{-3}	2.9×10^{-4}	7.8×10^{-2}	1.5×10^{-3}	99.3	0.2	0.5	0.5–40
BPS	-4.2×10^{-3}	2.5×10^{-5}	7.5×10^{-3}	1.4×10^{-4}	99.4	0.1	0.4	0.4–40
4-OH-BP	-1.0×10^{-2}	1.1×10^{-4}	5.1×10^{-2}	5.6×10^{-4}	99.8	0.1	0.3	0.3–40
BP-1	1.3×10^{-2}	7.9×10^{-5}	3.6×10^{-2}	4.2×10^{-4}	99.8	0.1	0.4	0.4–40
BP-2	1.8×10^{-3}	1.4×10^{-5}	6.4×10^{-3}	7.6×10^{-5}	99.7	0.1	0.4	0.4–40
BP-3	1.6×10^{-2}	1.7×10^{-4}	8.5×10^{-2}	9.0×10^{-4}	99.8	0.06	0.2	0.2–40
BP-6	2.6×10^{-3}	1.3×10^{-5}	9.8×10^{-3}	7.0×10^{-5}	99.9	0.06	0.2	0.2–40
BP-8	-2.7×10^{-3}	5.0×10^{-5}	4.7×10^{-2}	3.0×10^{-4}	99.8	0.06	0.2	0.2–40

a, intercept; s_a, intercept standard deviation; *b*, slope; s_b, slope standard deviation; R^2, determination coefficient; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range.

Table 4
Recovery assay, precision and trueness of target compounds in synthetic urine.

	Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	Recovery (%)	t_{calc}		Spiked (ng mL^{-1})	Found[a] (ng mL^{-1})	Recovery (%)	t_{calc}
MP	5	5.1 ± 0.1 (3.5)	102	2.14	**BPS**	2	2.1 ± 0.1 (6.3)	103	1.31
	120	123 ± 3 (4.7)	102	1.54		10	10.4 ± 0.7 (7.1)	104	1.71
	300	292 ± 14 (9.5)	98	0.81		40	38 ± 4 (9.7)	96	1.31
EP	5	5.0 ± 0.1 (4.5)	101	0.58	**4-OH-BP**	2	2.1 ± 0.1 (6.2)	104	2.02
	120	124 ± 5 (7.7)	103	1.29		10	10.3 ± 0.3 (5.5)	103	1.44
	300	295 ± 10 (7.3)	98	0.69		40	42 ± 3 (14.9)	104	0.87
IsPP	5	4.8 ± 0.2 (6.0)	96	2.16	**BP-1**	2	1.9 ± 0.1 (11.5)	96	1.16
	120	122 ± 1 (2.0)	101	2.08		10	10.0 ± 0.2 (4.1)	99	1.02
	300	299 ± 3 (2.3)	100	0.30		40	40 ± 2 (9.0)	99	0.17
PP	5	5.2 ± 0.2 (5.0)	103	1.80	**BP-2**	2	1.9 ± 0.1 (8.0)	97	0.97
	120	124 ± 5 (7.2)	103	1.31		10	9.8 ± 0.3 (6.1)	98	1.07
	300	304 ± 6 (3.8)	102	1.17		40	39 ± 2 (7.9)	97	1.09
IsBP	5	5.2 ± 0.1 (4.8)	103	1.99	**BP-3**	2	2.0 ± 0.1 (6.6)	100	0.19
	120	120 ± 2 (2.1)	100	0.04		10	10.0 ± 0.3 (5.0)	100	0.14
	300	304 ± 6 (4.0)	101	1.15		40	39 ± 2 (9.0)	98	0.56
BP	5	4.7 ± 0.2 (9.1)	94	1.98	**BP-6**	2	2.1 ± 0.1 (12.3)	104	0.99
	120	126 ± 7 (10.4)	105	1.37		10	10.6 ± 0.3 (5.8)	103	1.54
	300	299 ± 6 (4.2)	99	0.24		40	38 ± 2 (8.0)	96	1.72
BPA	2	1.9 ± 0.1 (6.6)	102	0.54	**BP-8**	2	2.0 ± 0.1 (5.7)	101	0.46
	10	10.5 ± 0.8 (7.3)	105	1.99		10	10.1 ± 0.2 (3.4)	101	1.40
	40	39 ± 2 (4.3)	98	1.73		40	39 ± 2 (10.7)	97	0.88

[a] Mean of 18 determinations ± confidence interval (Relative standard deviation, %).

Table 5
Method application to human urine samples.

Sample	Form	Concentration (ng mL^{-1})[a]								
		MP	**EP**	**IsPP**	**PP**	**BP**	**4-OH-BP**	**BP-1**	**BP-3**	**BPA**
Females										
S01	Free	11.6	1.5	ND	1.4	ND	ND	ND	ND	ND
	Total	1183	253	D	130	D	ND	7.7	ND	ND
S02	Free	5.1	D	D	0.9	0.5	3.4	ND	ND	ND
	Total	497	78	0.3	49	23	3.7	2.3	18	12
S03	Free	1.6	ND	ND	D	ND	ND	D	0.6	ND
	Total	9.5	17	ND	2.4	D	0.4	31	100	1.3
S04	Free	4.4	ND	ND	0.7	ND	D	ND	ND	ND
	Total	668	ND	0.4	97	ND	0.5	0.5	2.1	ND
S05	Free	1.4	ND	ND	0.3	D	D	ND	ND	ND
	Total	2.4	2.8	ND	0.5	D	3.6	1.7	D	ND
S06	Free	1.6	ND	ND	0.6	ND	D	ND	D	ND
	Total	36	0.8	0.4	11	0.5	D	0.7	6.2	ND
S07	Free	4.4	0.7	ND	0.3	ND	0.5	D	ND	ND
	Total	181	98	0.3	5.3	D	0.8	5.4	7.7	D
S08	Free	3.7	ND	0.2	0.8	ND	0.4	ND	ND	ND
	Total	45	0.8	0.3	2.9	D	1.3	2.2	6.2	0.5
S09	Free	0.8	ND	ND	ND	ND	ND	ND	ND	ND
	Total	33	14	ND	1.4	ND	0.6	0.8	1.3	1.1
S10	Free	1.1	ND	ND	0.3	ND	D	ND	ND	ND
	Total	112	49	0.4	10	ND	0.7	0.7	2.7	D
Males										
S11	Free	ND	ND	ND	D	ND	ND	ND	D	ND
	Total	19	0.3	ND	5.8	0.4	D	17	81	ND
S12	Free	0.4	ND	ND	D	ND	D	ND	ND	D
	Total	145	39	0.2	22	23	1.2	1.0	6.6	46
S13	Free	0.3	1.2	ND	ND	ND	D	ND	ND	ND
	Total	6.4	5.5	ND	1.0	0.3	0.4	0.9	5.1	D
S14	Free	1.6	ND	ND	0.7	ND	ND	ND	0.2	ND
	Total	24	0.3	ND	7.2	ND	0.8	ND	3.3	0.6
S15	Free	3.1	ND	ND	ND	ND	D	ND	ND	ND
	Total	9.3	9.9	ND	0.8	D	0.6	3.0	10	4.7
S16	Free	3.3	ND	ND	0.3	ND	0.5	ND	ND	ND
	Total	20	22	0.4	1.2	2.1	2.8	0.9	1.6	4.8
S17	Free	0.7	ND	ND	D	D	D	ND	ND	ND
	Total	3.9	2.0	ND	0.7	D	D	D	2.0	ND
S18	Free	1.4	ND	ND	1.6	D	D	ND	ND	ND
	Total	84	ND	ND	21	D	0.5	0.5	2.5	ND
S19	Free	ND	ND	ND	0.5	ND	0.3	ND	ND	ND
	Total	ND	ND	ND	1.4	D	0.4	ND	0.3	0.7
S20	Free	0.6	ND	ND	D	ND	0.3	ND	ND	ND
	Total	15	4.2	ND	3.7	ND	0.7	1.3	0.9	D

[a] Mean of 3 determinations; ND, not detected (< LOD); D, detected (> LOD and < LOQ).

optimization of two variables, studying one of them at three levels (in this case the volume of acetone) and the second one at five levels (in this case the volume of TCM). Spiked human pool urine at a concentration of 10 ng mL^{-1} of all compounds was used in this experiment.

Response surfaces of some target EDCs (one compound per family) are given in Fig. 1. In all cases, optimal extraction efficiencies were obtained at minimal volume of acetone, 0.50 mL, and maximal volume of trichloromethane, 0.75 mL.

3.2. Analytical performance

Due to a lack of EDCs free human urine samples, synthetic urine was used for calibration purposes [47]. This strategy has been previously applied for these purposes in the determination of different EDCs [27–29].

An eight concentration level calibration curve was built. Each level of concentration was made in triplicate. Calibration curves were constructed using analyte/surrogate peak area ratio *versus* concentration of analyte. Calibration graphs were made using SRM mode. For parabens, EP-$^{13}C_6$ at a concentration of 50 ng mL^{-1} was used as surrogate. BPA-d_{16}, at a concentration of 20 ng mL^{-1}, was used as surrogate for benzophenones and bisphenols.

In order to estimate the presence/absence of matrix effect, two calibration curves were obtained for each compound, one in distilled water and the other one in synthetic urine. The Student's *t*-test was applied in order to compare the calibration curves. First, the variances estimated as $S^2_{y/x}$ were compared by means of a Snedecor's *F*-test. The Student's *t*-test showed statistical differences among slope values for the calibration curves in all cases and consequently, the use of matrix-matched calibration was necessary. Table 3 shows the analytical parameters obtained.

3.3. Method validation

Validation in terms of linearity, sensitivity, accuracy (trueness and precision), and selectivity, was performed according to the US Food and Drugs Administration (FDA) guideline for Bioanalytical Method Validation [48].

3.3.1. Linearity

A concentration range for the minimal quantified amount (see Table 3) to 40 ng mL^{-1} for BPs, BPA and BPS, and to 300 ng mL^{-1} for PBs was selected. Linearity of the calibration graphs was tested using the determination coefficients (% R^2) and the *P*-values (% P_{lof}) of the *lack-of-fit* test [49]. The values obtained for R^2 ranged from 99.3 for BPA to 99.9% for IsPB, IsBP and BP-6, and P_{lof} values were higher than 5% in all cases. These facts indicate a good linearity within the stated ranges.

3.3.2. Limits of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) are two fundamental parameters that need to be examined in the validation of any analytical method to determine if an analyte is present in the sample. The LOD is the minimum detectable amount of analyte in the sample, while the LOQ is the minimum amount that could be quantified. In the present work, these parameters were calculated by taking into consideration the standard deviation of residual, $S_{y/x}$, the slope, *b*, of the calibration graphs and an estimate s_0 obtained by extrapolation of the standard deviation of the blank [50]. The LOD was 3 s_0 and the LOQ was 10 s_0. Found limits of quantification ranging from 0.2 to 0.5 ng mL^{-1}. These results are also summarized in Table 3.

3.3.3. Accuracy (precision and trueness)

Due to the absence of certified materials, in order to evaluate the trueness and the reproducibility of the method, a study with

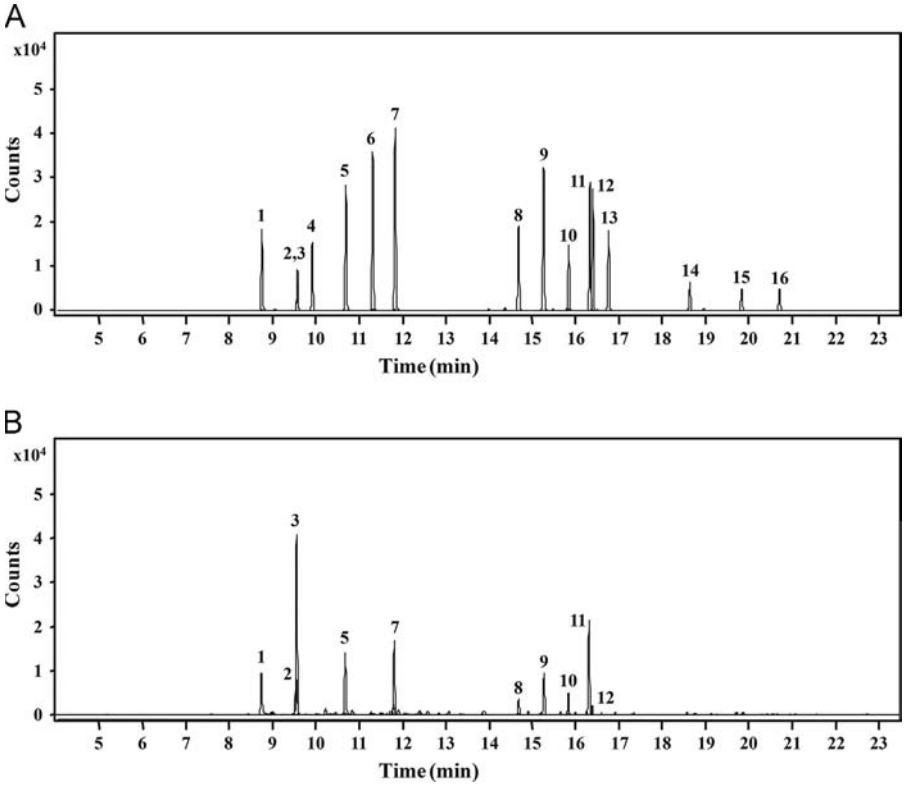

Fig. 2. SRM mode chromatograms: (A) a synthetic urine spiked sample (5 ng mL^{-1} of each studied analyte) and (B) a real urine human sample with enzymatic treatment. (1) MP; (2) EP; (3) EP-$^{13}C_6$; (4) IsPP; (5) PP; (6) IsBP; (7) BP; (8) 4-OH-BP; (9) BP-3; (10) BP-1; (11) BPA-d_{16}; (12) BPA; (13) BP-8; (14) BP-6; (15) BP-2; (16) BPS.

spiked synthetic urine samples, at three concentrations levels for each compound (2, 10 and 40 ng mL^{-1} for BPA, BPS and BPs; and 5, 120 and 300 ng mL^{-1} for PBs), was performed on six consecutive days. The precision was expressed as relative standard deviation, RSD, and the trueness was evaluated by a recovery assay. The precision and the trueness of the proposed analytical method are shown in Table 4.

Trueness was evaluated by determining the recovery of known amounts of the tested compounds in synthetic urine samples. Samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation in the standard calibration curve within the linear dynamic range and compared to the amount of analytes previously added to the samples. A recovery test (Student's *t*-test) was carried out. The results are also shown in Table 4. As calculated *P*-values calculated were > 0.05 (5%) in all cases, the null hypothesis appears to be valid, *i.e.*, recoveries are close to 100%.

Inter-day precision (expressed as relative standard deviation, RSD) was lower than 15%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation, which are considered $\leq 15\%$ of the actual value, except at the LOQ, which it should not deviate by more than 20%. The data (shown in Table 4) demonstrated that the proposed method is highly reproducible.

Precision and trueness data indicate that the methodology to determine the target compounds in human urine samples is highly accurate, and that the presence of co–extracted matrix components, which typically suppress the analyte signal in mass spectrometry, did not affect the performance of the method.

3.4. Method application

The validated method was applied to the determination of the amounts of free and total (free+conjugated) EDCs in 20 human urine samples from unknown men and women. All samples were analyzed in triplicate. The results obtained as mean of three determinations are summarized in Table 5. Fig. 2 shows the SRM chromatograms obtained, with transition used for quantification, for: (A) a spiked synthetic urine sample (5 ng mL^{-1} of each studied analyte); and (B) a real human urine sample (S13) with enzymatic treatment.

As it is shown in Table 5 and Fig. 3, MP, EP, IsPP, BP, 4-OH-BP, BP-1, BP-3 and BPA were detected and quantified in assayed

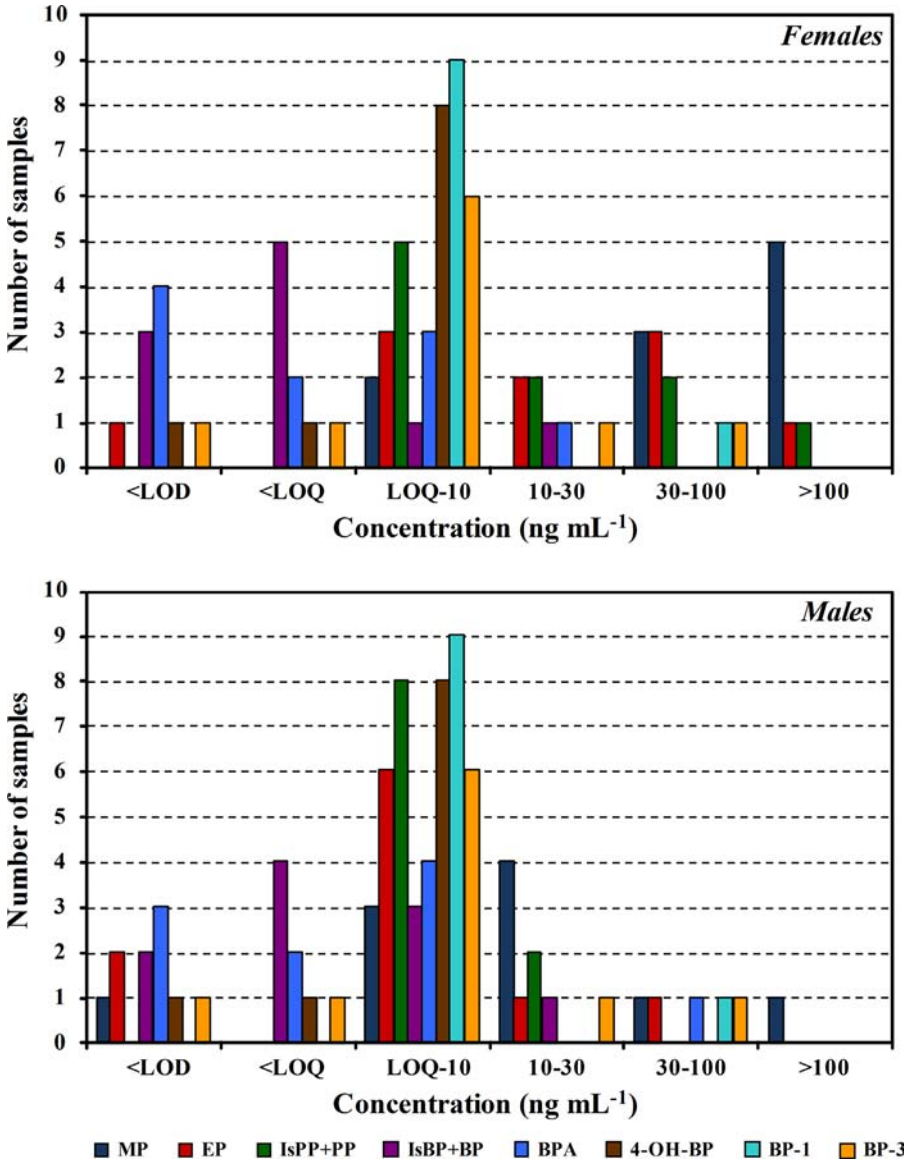

Fig. 3. Detection frequencies of total BPs, PBs and BPA in urine human samples.

samples. There were differences between free and conjugated (total less free) forms of BPs.

The conjugated form of 4-OH-BP was detected in almost all samples ($n=19/20$) and was quantified, at concentrations ranging from 0.3 to 3.6 ng mL^{-1}, in 80% of samples. But its free form was detected in 75% of samples and it was quantified, at concentrations ranging from 0.3 to 3.4 ng mL^{-1}, in 30% of samples. In the case of BP-3, its conjugated form was detected in almost all samples ($n=19/20$) and it was quantified in 90% of samples, at concentrations ranging from 0.3 to 99 ng mL^{-1}. Free form of BP-3 was detected in 20% of samples and it was quantified, at concentrations ranging from 0.2 to 0.6 ng mL^{-1}, in 10% of samples. For BP-1, its conjugated form was detected in 85% and it was quantified in 90% of samples, at concentrations ranging from 0.5 to 31 ng mL^{-1}. But free form of BP-1 was detected (not quantified) only in one them.

Regarding to parabens, the conjugated forms of MP and PP were detected and were quantified, at concentrations ranging from 1.0 to 1171 ng mL^{-1} for MP and from 0.2 to 128 ng mL^{-1} for PP, in most of the samples ($n=19/20$ and $n=20/20$, respectively). The free form of MP was detected and quantified, at concentration levels ranging from 0.3 to 11.6 ng mL^{-1}, in 80% of samples. The free form of PP was detected in 85% of samples and was quantified in 60% of samples at concentrations ranging from 0.3 to 1.6 ng mL^{-1}. In the case of EP, its conjugated form was detected and it was quantified in 85% of samples, at concentrations ranging from 0.3 to 251 ng mL^{-1}. But its free form was detected only in 20% of samples and it was quantified, at concentrations ranging from 0.7 to 1.5 ng mL^{-1}, in 15% of samples. For BP, its conjugated form was detected in 70% and was quantified only in 30% of samples, at concentrations ranging from 0.3 to 22.6 ng mL^{-1}. The free form BP was detected in 20% of samples and was quantified only in one them. The conjugated form of IsPP was detected in 45% of samples and was quantified, at concentrations ranging from 0.1 to 0.4 ng mL^{-1}, in 40% of samples. The free form of IsPP was detected in two samples and quantified only in one them.

Concentrations of total benzophenones and parabens in urine samples from females were significantly higher than those in males. This suggests higher exposures in females than in males, what can be attributed to higher usage of sunscreen products and also other cosmetic products containing UV filters by females.

Furthermore, there seems to be a relationship between the presence of BP-3 and BP-1 in the analyzed samples because in all samples that were present BP3, BP-1 was also detected. This fact suggests a possible conversion of BP-3 into BP-1 (see Section 1), so that the content of BP-1 may be due to human metabolism and not to a direct exposure.

Among the six parabens analyzed, MP and PP were the major compounds found in most urine samples. A significant linear relationship was found between urinary concentrations of MP and PP. This indicates concurrent exposure to these two compounds. EP also was detected frequently in urine, although their concentrations were lower than those of MP and PP. Concentrations and detection rate of IsPP and BP in urine were lower than those of EP.

In the case of BPA, its conjugated form was detected in 60% of samples and was quantified in 45% of samples at concentrations ranging from 0.5 to 46 ng mL^{-1}. But its free form was detected (not quantified) only in one them.

The present results, concentrations and detection rate of studied compounds in urine human, are in agreement with those recently reported by others authors [14,27,29,30]. Therefore, the method developed in this work is valid for the determination of BPs, PBs and bisphenols in human urine samples.

4. Conclusions

The identification and quantification of free and total concentrations of six parabens, six benzophenones and two bisphenols in human urine samples was successfully performed using the DLLME–GC–MS/MS method. The isolation of analytes from urine samples was accurately optimized and the procedure was validated. The proposed method has been used for determination of these compounds (free and total content) in samples collected from 20 randomly selected individuals (men and women). This is an analytical method that can be used in further studies for the determination of human exposure to EDCs.

Acknowledgments

This study was supported by the Regional Government of Andalusia (Project of Excellence no. P09-CTS-4470). The authors are grateful to University of Granada for the fellowship granted to F. Vela-Soria.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.05.016.

References

[1] B.A. Cohn, M.S. Wolff, P.M. Cirillo, R.I. Sholtz, Environ. Health Perspect. 115 (2007) 406–414.
[2] I.N. Damgaard, N.E. Skakkebaek, J. Toppari, H.E. Virtanen, H. Shen, K. W. Schramm, J.H. Petersen, T.K. Jensen, K.M. Main, Environ. Health Perspect. 114 (2006) 133–138.
[3] F. Giordano, A. Abballe, E. De Felip, A. di Domenico, F. Ferro, P. Grammatico, A.M. Ingelido, V. Marra, G. Marrocco, S. Vallasciani, I. Figa-Talamanca, Birth Defects Res. Part A – Clin. Mol. Teratol. 88 (2010) 241–250.
[4] H. Itoh, M. Iwasaki, T. Hanaoka, H. Sasaki, T. Tanaka, S. Tsugane, Sci. Total Environ. 408 (2009) 37–42.
[5] G. Toft, A. Rignell-Hydbom, E. Tyrkiel, M. Shvets, A. Giwercman, C.H. Lindh, H.S. Pedersen, J.K. Ludwicki, K. Lesovoy, L. Hagmar, M. Spano, G.C. Manicardi, E.C. Bonefeld-Jorgensen, A.M. Thulstrup, J.P. Bonde, Epidemiology 17 (2006) 450–458.
[6] O. Vasiliu, J. Muttineni, W. Karmaus, Hum. Reprod. 19 (2004) 506–512.
[7] Bisphenol-A Website. Document available on line at: ⟨http://www.bisphenol-a.org⟩ (accessed 10.12.13.).
[8] P.D. Bailin, M. Byrne, S. Lewis, R. Liroff, Public awareness drives market for safer alternatives: Bisphenol A market analysis report, Investor Environmental Health Network: Falls Church, VA, 2008. Document available on line at: ⟨http://www.iehn.org/documents/BPA%20market%20report%20Final.pdf⟩ (accessed 11.12.13.).
[9] A.M. Calafat, X. Ye, L.Y. Wong, J.A. Reidy, L.L. Needham, Environ. Health Perspect. 116 (2008) 39–44.
[10] M.G. Soni, I.G. Carabin, G.A. Burdock, Food Chem. Toxicol. 43 (2005) 985–1015.
[11] F.A. Andersen, Int. J. Toxicol. 27 (2008) 1–82.
[12] S. El Hussein, P. Muret, M. Berard, S. Makki, P. Humbert, Exp. Dermatol. 16 (2007) 830–836.
[13] Final report on the safety assessment of Benzophenones-1, -3, -4, -5, -9, and -11, Mary Ann Liebert, Inc., J. Am. College Toxicol, 2 1983, pp. 35–77.
[14] T. Kunisue, Q. Wu, S. Tanabe, K.M. Aldousa, K. Kannanc, Anal. Methods 2 (2010) 707–713.
[15] J.R. Byford, L.E. Shaw, M.G.B. Drew, G.S. Pope, M.J. Sauer, P.D. Darbre, J. Steroid Biochem. Mol. Biol. 80 (2002) 49–60.
[16] J.J. Prusakiewicz, H.M. Harville, Y. Zhang, C. Ackermann, R.L. Voorman, Toxicology 232 (2007) 248–256.
[17] J.M. Molina-Molina, A. Escande, A. Pillon, E. Gómez, F. Pakdel, V. Cavailles, N. Olea, S. Ait-Aissa, P. Balaguer, Toxicol. Appl. Pharmacol. 232 (2008) 384–395.
[18] T. Suzuki, S. Kitamura, R. Khota, K. Sugihara, N. Fujimoto, S. Ohta, Toxicol. Appl. Pharmacol. 203 (2005) 9–17.
[19] M. Takamiya, S. Lambard, I.T. Huhtaniemi, Reprod. Toxicol. 24 (2007) 265–275.
[20] C.A. Richter, L.S. Birnbaum, F. Farabollini, R.R. Newbold, B.S. Rubin, C. E. Talsness, J.G. Vandenbergh, D.R. Walser-Kuntz, F.S. vom Saal, Reprod. Toxicol. 24 (2007) 199–224.
[21] S. Oishi, Food Chem. Toxicol. 42 (11) (2004) 1845–1849.
[22] H. Jarry, J. Christoffel, G. Rimoldi, L. Koch, W. Wuttke, Toxicology 205 (2004) 87–93.

[23] S. Salian, T. Doshi, G. Vanage, Toxicology 265 (2009) 56–67.
[24] K.S. Kang, J.H. Che, D.Y. Ryu, T.W. Kim, G.X. Li, Y.S. Lee, J. Vet. Med. Sci. 64 (2002) 227–235.
[25] P. Berbel, J.L. Mestre, A. Santamaría, I. Palazón, Thyroid 19 (5) (2009) 511–519.
[26] S.L. Schantz, J.J. Widholm, D.C. Rice, Environ. Health Perspect. 111 (2003) 357–376.
[27] S.Y. Lee, E. Son, J.Y. Kang, H.S. Lee, M.K. Shin, H.S. Nam, S.Y. Kim, Y.M. Jang, G.S. Rhee, Bull. Korean Chem. Soc. 34 (2013) 1131–1136.
[28] M.S. Nahar, A.S. Soliman, J.A. Colacino, A.M. Calafat, K. Battige, A. Hablas, I.A. Seifeldin, D.C. Dolinoy1, L.S. Rozek, Environ. Health 11 (2012) 1–8.
[29] A.M. Calafat, X. Ye, L.Y. Wong, A.M. Bishop, L.L. Needham, Environ. Health Perspec. 118 (2010) 679–685.
[30] C. Liao, K. Kannan, Environ. Sci. Technol. 46 (2012) 5003–5009.
[31] L. Wang, Y. Wu, W. Zhang, K. Kannan, Environ. Sci. Technol. 47 (2013) 2069–2076.
[32] L. See Wang, K. Kannan, Environ. Sci. Technol. 47 (2013) 12532–12538.
[33] A.G. Asimakopoulos, N.S. Thomaidis, K. Kannan, Sci. Total Environ. 470–471 (2014) 1243–1249.
[34] I. Jiménez-Díaz, F. Vela-Soria, A. Zafra-Gómez, A. Navalón, O. Ballesteros, N. Navea, M.F. Fernández, N. Olea, J.L. Vílchez, Talanta 84 (2011) 702–709.
[35] F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, M.F. Fernández, N. Olea, A. Navalón, Anal. Methods 3 (2011) 2073–2081.
[36] F. Vela-Soria, I. Jiménez-Díaz, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, A. Navalón, J.L. Vílchez, M.F. Fernández, N. Olea, Talanta 85 (2011) 1848–1855.
[37] L.P. Melo, M.E.C. Queiroz, Anal. Methods 5 (2013) 3538–3545.
[38] M. Schlumpf, K. Kypke, M. Wittassek, J. Angerer, H. Mascher, D. Mascher, C. Voekt, M. Birchler, W. Lichtensteiger, Chemosphere 81 (2010) 1171–1183.
[39] X. Ye, A.M. Bishop, L.L. Needham, A.M. Calafat, Anal. Chim. Acta 622 (2008) 150–156.
[40] M. Rezaee, Y. Assadi, M.R. Milani-Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A 1116 (2006) 1–9.
[41] C. Bosch-Ojeda, F. Sánchez-Rojas, Chromatographia 74 (2011) 651–679.
[42] H. Yana, H. Wanga, J. Chromatogr. A 1295 (2013) 1–15.
[43] M. Vosough, N.R. Mojdehi, A. Salemi, J. Sep. Sci. 35 (2012) 3575–3585.
[44] I. Tarazona, A. Chisvert, A. Salvador, Talanta 116 (2013) 388–395.
[45] F. Vela-Soria, O. Ballesteros, I. Rodríguez, A. Zafra-Gómez, L. Ballesteros, R. Cela, A. Navalón, Anal. Bioanal. Chem. 405 (2013) 7259–7267.
[46] F. Vela-Soria, O. Ballesteros, A. Zafra-Gómez, L. Ballesteros, A. Navalón, Talanta 121 (2014) 97–104.
[47] K.G.W. Inn, D. McCurdy, L. Kuruvilla, N.M. Barss, R. Pietrzak, E. Kaplan, W. Inkret, W. Efurd, D. Rokop, D. Lewis, P. Gautier, R.T. Bell, J. Radioanal. Nucl. Chem. 249 (2001) 121–131.
[48] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), 2001.
[49] Analytical Methods Committee, Analyst 119, 1994, pp. 2363–2366.
[50] L.A. Currie, Anal. Chim. Acta 391 (1999) 127–134.