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Determination of endocrine disruptors and endogenic androgens and estrogens in rat serum by high-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

To simultaneously measure some targeted endocrine disruptors and several forms of sex hormones in rat serum, an accurate analytical procedure was developed. First, a comparison between a polymeric-based solid-phase extraction (SPE) and a micro-extraction by packed sorbent was performed to choose the optimal method to extract and concentrate the analytes: bisphenol A, atrazine, vinclozolin metabolite, testosterone, androstenedione, estrone, estradiol, estrone-sulfate and glucuronide and estradiol-sulfate and glucuronide. The analyses were then performed by high-performance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) with electrospray ionisation in positive and negative modes. The protocol based on SPE was validated using the ICH/2005 guidelines. The validation demonstrated good performance in terms of linearity ($R^2 > 0.99$), recovery (71–90%) and repeatability (relative standard deviation: 1–18%). The method was sensitive with LOQ comprised between 0.1 and 0.4 ng/ml for androgens and between 0.098 and 10.2 ng/ml for estrogens. The results obtained on the serum of rats exposed to the targeted endocrine disruptors showed the suitability of this analytical strategy.

1. Introduction

Endocrine disrupting chemicals (EDCs) are substances that can cause adverse effects by interfering with the body's hormones or chemical messengers. The number of substances that have been identified as potential EDCs increases every year and these EDCs belong to different families of chemicals: dioxins, polybromodiphenylethers, phthalates, parabens, alkylphenols, pesticides, etc. In 2007, the European Commission published a document on the implementation of the "Community Strategy for Endocrine Disrupters – a range of substances suspected of interfering with the hormone systems of humans and wildlife". A total of 575 substances were investigated of which 320 showed evidence or potential evidence for endocrine disrupting effects [1]. The availability of validated exposure data is a critical component for assessing the causal relationships between exposure to EDCs and health effects [2]. Although more and more data are available on these compounds, their mechanisms of action are not yet clear [3] and warrant further investigation.

One potential target for EDCs is steroidogenesis, which allows a finely tuned sex hormone balance. Interactions between chemicals and key enzymes involved in steroidogenesis may dramatically alter the steroid hormone balance and endocrine function. For example it has been put in evidence that an altered estrogen-to-androgen balance is associated with the development of various diseases [4,5].

The utility of the laboratory rat as an animal model for endocrine screening is evident in the literature [6]. Most regulatory tests available to study EDC toxicity have been developed in these animals. These *in vivo* tests integrate metabolism and feedback loops; these assays typically look at relevant integrated toxicity endpoints, such as impact on fertility [7]. The extrapolation of effects in the rat to humans and other vertebrate species can be performed by the use of the wealth of information that is available in the literature [6].

To better understand the impact of EDCs on the hormonal system, it is necessary to develop highly sensitive analytical methods able to quantify simultaneously the endogenous hormones and EDCs on biological matrices such as plasma, serum, whole blood, urine or tissues. Among these matrices, serum is often used because it includes all proteins not used in coagulation, all the electrolytes, antibodies, antigens, as well as hormones and any exogenous

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substances (e.g., drugs and micropollutants). Most analytical methods on serum are developed towards a single family of chemicals. They can have two different objectives: the measurement of steroid concentrations after an exposure to EDCs or the measurement of EDCs themselves. Methods to analyse steroids in serum [8,9] are more numerous than methods to analyse xenobiotics. Analytical strategies are commonly based on solid-phase extraction (SPE) or liquid–liquid extraction (LLE) [10] followed by gas chromatography coupled with mass spectrometry (GC–MS) or liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). GC–MS is now less common than LC–MS/MS because it requires a derivatization step [11]. Compared to LLE, an advantage of the SPE technique is that it consumes less solvent. A miniaturised version of SPE, called microextraction by packed sorbent (MEPS), has recently gained attention in the field of biological matrix analysis [12–15]. It is a technique developed in 2003 by Abdel-Rehim [16] that combines the sample processing, extraction and injection steps into a fully automated fashion as an at-line sampling/injecting device to GC or LC. In MEPS, a sorbent is placed within a special needle or barrel, acting as a cartridge. MEPS is a miniaturised format of SPE, meaning that the sample volume and the organic solvent consumption are significantly reduced. Although many analytical methods for steroids have been reported, steroid conjugates are rarely analysed. Labrie et al. [17] analysed human serum glucuronide derivatives of androgens with SPE on C18 cartridges and DHEA sulfate with SPE on Oasis HLB, both followed by LC–MS/MS. In 2007, Xu et al. [18] determined steroid conjugate concentrations by the subtraction of total estrogens after enzymatic hydrolysis and the level of free estrogens. Qin et al. published two analytical methods with the same extraction based on SPE followed by two different separations, LC–MS/MS with hydrophilic interaction liquid chromatography (HILIC) separation for the determination of estrone (E1), estradiol (E2), and estriol (E3) conjugates in human urine [19] and a column-switching reversed phase HILIC LC–MS/MS method for the determination of the same free estrogens and their conjugates in river water [20]. Caron et al. [21] analysed glucuronide conjugates of E1, E2 and their methoxy metabolites in serum using two extractions, SPE and LLE, followed by LC–MS/MS. Finally, extractions based on SPE were used by Strahm et al. [22] to analyse androgen sulfoconjugates in human urine and Giton et al. [23] to determine estrone and estrone sulfate (E1S) in human plasma.

In this context, the goal of this work was to develop a fast and sensitive multi-residue analysis for four EDCs and endogenous hormones and their conjugates in rat serum.

Due to its ubiquitous presence in the environment, bisphenol A (BPA) is one of the most studied EDC. BPA was clearly defined as an estrogenic agent due to its capacity to bind estrogen receptor [24,25]. Another family of compounds that is gaining the attention of environmentalists and health professionals are pesticides [26]. One of the most commonly detected pesticides in the environment is atrazine (ATZ), which, among other effects, alters male reproductive tissues [27]. The compound 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), which is a major metabolite of the pesticide methoxychlor (MXC), exhibits estrogenic, anti-estrogenic and anti-androgenic capacities [25,28]. Vinclozolin (VCZ) is unstable in aqueous media and presents anti-androgenic properties through two metabolites, 2-[[(3,5-dichlorophenyl)-carbamoyl] oxy]-2-methyl-3-butenoic acid (V M1) and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (V M2) [29].

Free and conjugated forms of the main sexual steroid pathways were included in this work. E1 and E2 are the major estrogens involved in the development and maintenance of the female phenotype and pregnancy. Moreover, they have important roles in male via aromatization of testosterone (T) within tissues. Similarly T is a natural androgen responsible for the development and maintenance of the male phenotype. Meanwhile androstenedione (A) is an intermediate step in the biochemical pathway that produces T, E1 and E2.

To resume, the objective was to develop a method based on LC–MS/MS technique to analyse simultaneously four endocrine disruptors and endogenic androgens and estrogens as well as their conjugates in rat serum with a minimum numbers of steps. Two one-step extractions were chosen and compared: the SPE conventional technique that has proven to extract biological fluids and MEPS which is a new embodiment for miniaturised SPE in the field of sample handling and preparation.

2. Materials and methods

2.1. Chemicals and reagents

(T), (E1) and (E2) were greater than 98% pure and were purchased from Sigma–Aldrich (Saint-Louis, Missouri, USA). (A) was 99% pure and was purchased from Fluka (Buchs, Switzerland). Conjugated standard were more than 30% pure (70% of preservatives) and were from Sigma–Aldrich: estrone 3-(β-D-glucuronide) sodium salt (E1G), β-estradiol 3-(β-D-glucuronide) sodium salt (E2G), estrone 3-sulfate sodium salt (E1S) and β-estradiol 3-sulfate sodium salt (E2S). (ATZ) and (MXC) were Pestanal-quality and were from Fluka. (BPA) and (HPTE) were 99% pure and were from Sigma–Aldrich. (VCZ) and (V M2) were purchased from Cayman Chemical (Ann Arbor, Michigan, USA) and were 99.9% pure.

All deuterated standards, used with a concentration of 30 ng/mL as methodological performance controls, were of 98% pure (or more) and were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). These included 17β-estradiol-2,4-d_2 (E2-d_2) used for the control of E2, estrone-2,4-d_2 (E1-d_2) for E1, testosterone-1,2-d_2 (T-d_2) for T, 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7 (A-d_7) for A, 17β-estradiol-2,4,16,16-d_4 3-sulfate sodium salt (E2S-d_4) for E1S, E2S, E1G and E2G, bisphenol A -2,2',6,6'-d_4 (BPA-d_4) for BPA, HPTE and V M2 and finally atrazine-d_5 (ATZ-d_5) for ATZ.

A solution of 500 ng/mL of ethoxyphenacetin-^{13}C (PhC13), used as the injection control, was 99% pure and was purchased from ISOTEC™ (Miamisburg, Ohio, USA).

Methanol (MeOH) and hexane (HEX) were HPLC grade, and acetonitrile (ACN) was LC–MS grade. All solvents were obtained from Sigma–Aldrich. Ultra-pure water was obtained from a MilliQ device from Millipore (Billerica, Massachusetts, USA).

The stock solutions were prepared in MeOH then stored in a freezer at −23 °C for 6 months. The working solutions were prepared daily by mixing stock solutions in ultra-pure water.

2.2. Sample collection and preparation

Young male rat serum pools were obtained from INERIS (Institut National de l'Environnement industriel et des RISques) (Verneuil-en-Halatte, France) and were used for the tests, assays and validation procedures. A total of 130 male and 130 female adult Sprague–Dawley rats (certified virus free, Hla: (SD) CVF) purchased from Janvier (Le Genest-Saint-Isle, France) were chosen for the *in vitro* study. The rats were subjected to a 12 h light and 12 h dark cycle and received food and water *ad libitum*. All of the procedures were reviewed and approved by the Institutional Animal Care and Use Committee of INERIS. Rats were sacrificed by a lethal intraperitoneal pentobarbital injection after a two-week daily gavage administration of ATZ, BPA, MTX or VCZ. Whole blood samples were collected and centrifuged at 3000 g for 15 min at 4 °C. The supernatant were then stored at −20 °C until extraction and analysis. It was kept less than 6 month in these conditions.

Before SPE and MEPS extractions, the serum (1 ml) was buffered with 0.5 ml of acetate buffer (pH 5.2) and 30 μl of IS solution was added; then, the mix was sonicated for 20 min at 50 °C so as to break the bonds with proteins. The mixture was then centrifuged for 5 min at 15,000 g to remove proteins. This procedure severs the links between proteins such as SBP (steroid binding protein) but does not affect the conjugated forms.

2.3. Solid phase extraction

Various polymeric solid reversed phase media were tested to select the best extraction phase. Oasis® HLB (Waters, Saint-Quentin en Yvelines, France) is a spherical reversed-phase (60 mg/3 ml, particle size 30 μm, pore size 80 Å) composed of two monomers: hydrophilic N-vinylpyrrolidone and lipophilic divinylbenzene. SampliQ® Optimized Polymer Technology from Agilent Technologies (Santa Clara, California, USA) (OPT 60 mg/3 ml, particle size 30 μm, pore size 60 Å) is a spherical polyamide-polymer resin. Chromabond® HR-X from Macherey–Nagel (Düren, North Rhine-Westphalia, Germany) (60 mg/3 ml, particle size 85 μm, pore size 55–60 Å) is a spherical hydrophobic polystyrene–divinylbenzene adsorbent resin. Chromabond® EASY from Macherey–Nagel (60 mg/3 ml, particle size 80 μm, pore size 50 Å) is a polar modified polystyrene–divinylbenzene copolymer with a weak anion exchanger. Interchrom (Interchim, Montluçon, France) Atoll™ ATH (60 mg/3 ml; particle size 75 μm; pore size 70 Å) and 30ATH (same values except particle size 30 μm) have the same characteristics as Chromabond® EASY.

The SPE protocols were based on previous work [30]. SPEs were conducted using Rapid Trace instruments from Biotage (Uppsala, Sweden). Several polymer-based SPE cartridges were compared following the same preparation procedure.

The cartridges were conditioned with 5 ml of MeOH followed by 5 ml of acetate buffer (pH 5.2) at a 5 ml/min flow rate. The buffered serum (1.5 ml) was loaded at 1 ml/min onto the cartridge then the phase was washed with 3 ml of water and 2 ml of hexane at 4 ml/min. The elution was performed using 3 × 1 ml of MeOH at 1 ml/min. The extract was evaporated to dryness at 50 °C under a stream of nitrogen and then reconstituted in 50 μl of PhC13 (0.5 μg/ml) in an 80/20 water/ACN v/v solution for LC–MS/MS analysis. This internal standard can be used in both positive and negative modes to control the injection.

No carry-over of EDCs was detected by applying the extraction procedure to a blank sample, as tested on every solid polymer-based phase.

2.4. Microextraction by packed sorbent

MEPS was performed using a 50-μl eVol® hand-held automated analytical syringe from SGE Analytical Science (Ringwood, Victoria, Australia). Different sorbents (C8, C18 and M1 which is a mix of C8 and Strong Cation Exchange phases – C8/SCX) with mean particle sizes of 45 μm and pore sizes of 60 Å were tested. The Barrel Insert Needle (BIN) contained 4 mg of sorbent, and its capacity was 8 μl.

Before first time use, the sorbent was conditioned with 50 μl of MeOH followed by 50 μl of water at 7 μl/s.

The optimised protocol consisted of multiple loads (3 × 50 μl) of samples into the MEPS syringe containing C18 phase. The solid phase was then washed with 2 × 50 μl H_2O. Analytes were desorbed by 25 μl of MeOH/H_2O (95/5; v/v) and elution was finished with 25 μl of ACN. All steps were conducted at a flow rate of 1.7 μl/s. The phase washes were performed using 50 μl of ACN, 2 × 50 μl of HEX and 5 × 50 μl of MeOH, followed by 5 × 50 μl of H_2O at 7 μl/s.

The extract was evaporated to dryness at 50 °C under a stream of nitrogen and then reconstituted in 50 μl of PhC13 (0.5 μg/ml) in an 80/20 water/ACN v/v solution for the LC–MS/MS analysis.

No carry-over of EDCs was detected by application of the extraction procedure to a blank sample.

2.5. LC–MS/MS analysis

An ABSciex 3200 QTRAP with electrospray ionisation (ESI) in positive mode coupled to an Agilent 1200 LC, which includes a binary pump, column temperature control and auto sampler, was used. To obtain higher sensitivity in the negative mode, an ABSciex 5500 QTRAP with ESI coupled to an Agilent 1290 LC, which includes a quaternary pump, column temperature control and thermostated autosampler, was also tested. In the negative and positive modes, respectively, the source temperatures were 400 and 600 °C, and the ion spray potentials were – 4500 and 5500 V. ABSciex Analyst 1.5.1 software (Framingham, Massachusetts, USA) provided data collection and instrument control.

The analytical column was an Agilent ZORBAX Eclipse Plus C18 (1.8 μm, 50 mm length and 2.1 mm i.d). A pre-filter (Agilent frit 2 mm 0.2 μm) was installed upstream of the column. The injection volume was 5 μl, and the flow rate was 0.3 ml/min. The mobile phase (A) was 0.01 mM ammonium acetate in water, and the mobile phase (B) was acetonitrile.

In the negative mode, the gradient conditions were 90% of (A), ramped using a linear gradient to 33.5% (A) over 5 min, then ramped to 0% of (A) from 5 to 6 min and held until 11 min. In the positive mode, the gradient profile started at 75% of (A), decreased linearly to 33.5% in 4 min, then ramped to 0% of (A) in 1 min and held 10 min. A 10 min equilibration was performed before every run to give total run times of 21 min in the negative mode and 20 min in the positive mode.

2.6. Matrix spiking, matrix effect and validation

The validation method combining the SPE sample preparation protocol and the LC–MS/MS analysis (with 3200 QTrap) was based on the ICH Directive [31] to evaluate the linearity, the extraction recovery, the repeatability, the reproducibility, the limit of detection (LOD) and the limit of quantification (LOQ) as well as to conclude about reliability and efficiency. The implemented validation plan was performed on 9 points: 3 replicates of 3 concentration levels over 3 days.

Some hormones are naturally present in rat serum at various concentrations. To obtain a constant concentration for the validation assays, 15 rat serum samples were pooled and homogenised. Thus, all of the spiked and non-spiked samples were obtained from the same matrix, and the validation was performed from this pool. Targeted natural endogenous hormones (A; T; E1; E2; E1-S and –G; E2-S and –G) were previously determined by standard additions. As a consequence of the non-blank pool, LODs, LOQs and reporting limits were estimated based on signal-to-noise ratios and comparisons with spiked samples and standard solutions of each molecule (EDCs, free and conjugated hormones).

The analytes were identified by both their chromatographic characteristics and their multiple reaction monitoring (MRM) specific fragmentation. Indeed, we compared the specific retention times of the analytes, two of their characteristic ion transitions and the specific ratios of the intensities of their product ions (deviation <20% with respect to analytical standards ratios) with standards. Data processing was performed with the software Analyst (version 1.5.1).

To test the method linearity, it was necessary to establish a range of concentrations for each compound due to the different

compound sensitivities. Thus, the calibration curve for each analyte was based on five concentration levels. The validation criteria for linearity were based on correlation coefficients R^2 greater than 0.99.

Recovery and repeatability were evaluated on three levels of concentrations (low, middle and high corresponding to the second, the third and the fifth calibration points, respectively) presented in Table 1. Recoveries were determined by the analysis of pool samples spiked at the different concentration levels then extracted and comparison of the results to those of serum extracts spiked after the pool extraction. The repeatability is associated with the intra-day precision. To estimate it, the sample was spiked, extracted and analysed under the same conditions by the same operator and on the same day. Each concentration level was repeated three times and the repeatability was expressed as the relative standard deviation (RSD, expressed as percentage) of those replicates. In order to fulfil the ICH requirements, recoveries were expected to be comprised between 70% and 120% and to be repeatable.

The reproducibility was assessed at the same levels of concentrations as used for repeatability (Table 1), over three days using two different operators (operator 1 on the first two days, operator 2 on the last day). The reproducibility (or inter-day precision) was also expressed as the percentage of RSD.

Matrix effect (ME), such as enhancement or inhibition of analytical signals, is commonly observed during LC–MS/MS analyses using electrospray ionisation. To evaluate this ME, the samples were spiked after extraction, and the corresponding signal areas ($S_{extract}$) were compared with those obtained with standards at the same concentration in solvent ($S_{solvent}$). Matrix effects were then calculated according to the following equation: $ME_{\%} = (A_{extract}/A_{solvent} - 1) \times 100$.

3. Results and discussion

LC–MS/MS was chosen for the analysis as it permits the analysis of all molecules, without deconjugate the sulfo- and glucuro-conjugated hormones. A minimum number of steps and simultaneous extractions were required; therefore, a method without hydrolysis and derivatisation was investigated. Two one-step extractions were chosen and compared: the SPE conventional technique that has proven to extract biological fluids and MEPS which is a new embodiment for miniaturised SPE in the field of sample handling and preparation.

3.1. LC–MS/MS optimisation

The MS/MS conditions were optimised using direct infusion into the ESI source in both modes to determine the two most sensitive and selective multiple reaction monitoring (MRM) transitions of each analyte, one for quantification and a second for confirmation. Table 2 presents the retention times, MRM transitions and transition ratio, declustering potential and collision energy for each analyte and performance standard.

Several mobile phases were tested for the chromatographic separation. MeOH and ACN were first compared. ACN provided the most resolving separation, especially on the couples estrone–estradiol and testosterone–androstenedione and on the conjugated forms.

We compared formic acid (0.1% vol) and ammonium acetate (0.1 mM) solutions as aqueous phase. Formic acid showed ion suppression in negative mode, whereas ammonium acetate did not affect the three analytes in the positive mode and improved signals in the negative mode. Hence, ammonium acetate solution and acetonitrile were chosen as solvents.

Several ammonium acetate concentrations (0.01, 0.1 and 1 mM) were assayed to obtain a better resolution on conjugated forms. The 0.1-mM solution permitted the best compromise between ionisations and resolution of sulfated and glucuronidated forms.

Finally, the ramp was refined to the values described in Section 2.4.

3.2. SPE optimisation

First, polymeric and silica-based phase cartridges were compared. These cartridges included the Oasis HLB cartridge (60 mg/3 ml, particle size 30 μm, pore size 80 Å) and the Strata-C18 (200 mg/3 ml, particle size 55 μm, 70 Å) (results not shown). Extraction with the Oasis HLB cartridges presented better recoveries, especially for the conjugated forms. Hence, polymeric phases were preferred.

Then different polymeric phases were compared. These included the Waters Oasis® HLB, the Agilent SampliQ® OPT, the Interchrom Atoll® ATH and 30ATH and the Macherey–Nagel Chromabond® HR-X and EASY. The same extraction procedure, as described in Section 2.3, was applied for each phase. Extractions were first applied to 1-ml spiked water samples (MilliQ water). The analyte concentrations were 1.5 μg/ml, except for BPA and ATZ which were 0.75 μg/ml.

For a greater simplicity, Fig. 1 shows the normalised signals obtained with every phase and each analyte. The 100% signal was the highest intensity for all of the assays. The EASY phase, which includes a weak anion exchanger, was the least effective with conjugated forms giving recoveries of 13%, 15%, 23% and 26% for E1S, E2S, E1G and E2G, respectively, but gave one of the best recoveries with free hormones. The 30ATH phase was the least effective assay on free hormones. The recoveries obtained with OPT, ATH or HRX were similar and equal or lower than the HLB phase. For these reasons, Oasis HLB phase was chosen.

3.3. MEPS optimisation

In a recent tutorial dealing with the use of MEPS for biological fluid [32], the author indicates that the speed of the sample through the sorbent can be ranged from 10 to 20 μL/s for better interactions between analyte and sorbent. This range of speeds was observed for all tests.

As well, different MEPS phases had to be tested before any comparisons could be made between the two methods.

The first protocol consisted of a multiple load (5×50 μl) of serum sample into the syringe containing the adsorbent. The solid phase was then washed with 2×50 μl of H_2O. Analytes were desorbed by 25 μl of MeOH/H_2O (95/5 v/v). Extractions were performed on spiked samples. Analyte concentrations were 1.5 μg/ml, except for BPA and ATZ which were 0.75 μg/ml.

Fig. 2 shows the normalised signals observed with each phase on each analyte. The 100% signal was the most intense of each

Table 1
Concentration levels used for the calibration, in ng/ml in serum.

Analyte	Low level	Middle level	High level
ATZ	0.3	1.1	5.0
A	1.2	4.1	19.0
T	0.3	1.1	5.0
BPA	9.3	32.2	150.0
V M2	0.2	0.8	3.5
HPTE	57.5	199.0	923.0
E1	4.9	16.8	77.8
E2	19.9	69.0	320.0
E1G	8.9	30.7	142.0
E1S	0.3	1.0	4.6
E2G	29.4	102.0	472.0
E2S	0.3	1.0	4.6

Table 2
Analytes and controls MS/MS conditions (uncolored lines present MRM transitions used for qualification, gray-coloured lines for quantification; Prec = Precursor ion; Prod = Product ion; DP: Declustering Potential; CE: Collision Energy).

Mass detection mode	Analytes/controls	Prec (m/z)	Prod (m/z)	DT (ms)	DP (V)	CE (V)	Transition ratios	Retention times (min)
Positive 3200 QTRAP	Atrazine (ATZ)	216.2	174.1	30	41	23	2.6 ± 0.1	4.9 ± 0.2
		216.2	104.0	30	41	39		
	Testosterone (T)	289.3	97.1	30	46	31	1.0 ± 0.1	5.8 ± 0.2
		289.3	109.1	30	46	35		
	Androstenedione (A)	287.3	97.2	30	51	33	1.2 ± 0.1	6.1 ± 0.2
		287.3	109.1	30	51	35		
	ATZ d_5	221.2	179.2	30	36	27		4.9 ± 0.2
	T-d_2	291.3	99.1	30	46	29		5.8 ± 0.2
	A-d_7	294.3	100.1	30	46	31		6.1 ± 0.2
	Ethoxyphenacetine C13	181.1	109.9	30	36	27		1.6 ± 0.2
Negative 5500 QTRAP	Estradiol 3-glucuronide (E_2G)	447.1	112.9	15	−115	−30	1.5 ± 0.4	2.2 ± 0.2
		447.1	271.1	15	−115	−58		
	Estrone 3-glucuronide (E_1G)	445.1	113.0	15	−50	−26	1.4 ± 0.4	2.4 ± 0.2
		445.1	269.1	15	−50	−54		
	Estradiol 3-sulfate (E_2S)	351.1	271.1	15	−105	−54	4.6 ± 1.0	2.8 ± 0.2
		351.1	79.9	15	−105	−76		
	Estrone 3-sulfate (E_1S)	349.1	269.1	15	−130	−46	1.6 ± 0.1	3.0 ± 0.2
		349.1	145.0	15	−130	−74		
	Bisphenol A (BPA)	226.9	133.0	15	−115	−34	9.8 ± 1.1	3.9 ± 0.2
		226.9	116.9	15	−115	−66		
	17B Estradiol (E_2)	271.0	183.2	15	−55	−56	1.6 ± 0.1	4.2 ± 0.2
		271.0	144.8	15	−55	−60		
	HPTE	314.9	242.9	15	−30	−8	1.3 ± 0.2	4.5 ± 0.2
		314.9	279.0	15	−30	−6		
	Estrone (E_1)	268.9	145.1	15	−130	−50	7.7 ± 0.6	4.6 ± 0.2
		268.9	159.0	15	−130	−48		
	Vinclozoline M2 (V M2)	258.0	159.8	15	−65	−24	44.7 ± 3.9	5.0 ± 0.2
		258.0	34.9	15	−65	−66		
	E_2S d_4	355.1	275.2	15	−115	−50		2.8 ± 0.2
	BPA d_4	231.1	216.1	15	−80	−26		4.0 ± 0.2
	E_2 d_2	273.0	146.8	15	−65	−50		4.3 ± 0.2
	E_1 d_2	271.1	147.0	15	−120	−50		4.6 ± 0.2
	Ethoxyphenacetine C13	179.0	148.6	15	−30	−18		2.7 ± 0.2

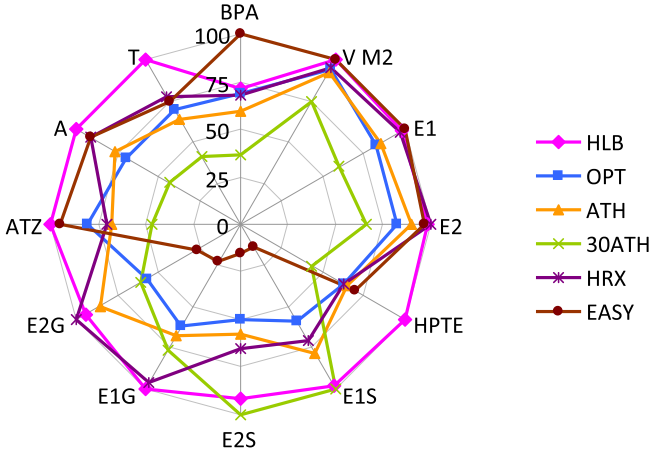

Fig. 1. Normalised signals obtained after SPE with various phases (HLB, OPT, ATH, 30ATH, HRX and EASY). Extractions performed with 1-ml of water samples; concentrations 1.5 µg/ml except for BPA and ATZ 0.75 µg/ml.

assay. As shown, each 100% signal, except for BPA (75% signal), was obtained with the C18 phase. MEPS was rarely developed to analyse steroids in biological fluids [15]. A C18-MEPS protocol was evaluated for measuring steroid hormone-related compounds in urine [31]. Most recoveries were comprised between 60% and 98% in the case of model hydroxylated steroid compounds. Our findings confirm the effectiveness of this phase on free steroid compounds and demonstrate its efficiency on multiple residues.

This phase was consequently chosen to extract serum samples, and the optimisation was performed. We noticed that a second 25-µl elution with acetonitrile was beneficial to the extraction. With serum samples, we observed that a 5-time load (5 × 50 µl) clogged up the BIN faster than a 3-time load. As no significant differences were observed regarding the matrix effects, a 3-time load was chosen for the next step.

3.4. Comparison between SPE and MEPS methods

First, optimised protocols explained in the paragraph *Sample collection and preparation* were applied to three spiked serum samples, three raw serum samples and three spiked water samples. The two first batches were used to calculate the recoveries. Raw serum extracts were spiked after extraction at expected levels of concentration to assimilate recoveries to 100%. Fig. 3 represents the recoveries obtained with both methods.

Regarding SPE, the serum samples were spiked at a concentration of 2 µg/ml for all analytes. The expected concentrations in the extracts were 40 µg/ml due to the final 20 × concentration. In the case of MEPS, the serum samples were spiked at a concentration of 600 ng/ml. Due to the final 3 × concentration, the extracts were spiked at 1.8 µg/ml.

No significant differences are observed between the SPE and MEPS results, regarding free hormones. The recoveries were approximately 60%, except for V M2 which was approximately 80% and HPTE which was 75% with SPE and 31% with MEPS. The difference between the two techniques is pointed out by the conjugated hormones. E1-S and E2-S, respectively, had recoveries of 16% and 12% on MEPS and 63% and 60% on SPE. E1-G and E2-G, respectively, had recoveries of 39% and 29% on MEPS and 74% and 76% on SPE.

Second, spiked water and serum samples permitted observation of the matrix effects, accounting for the extraction. This effect,

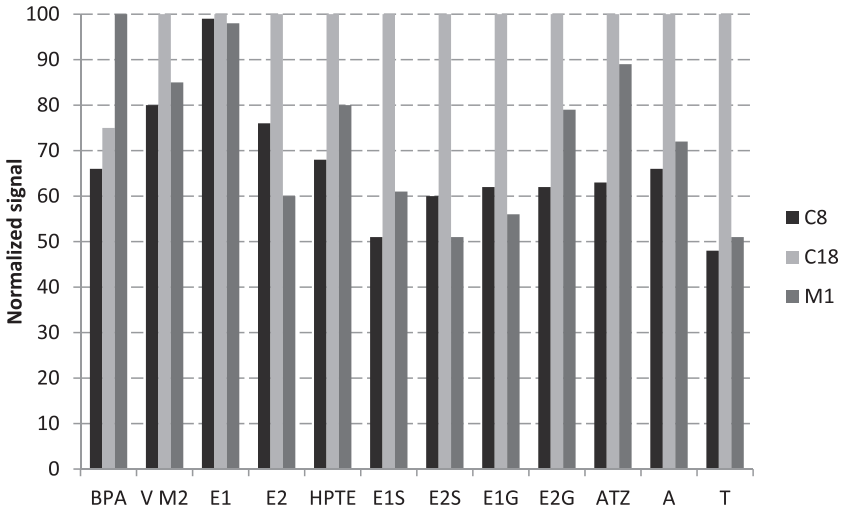

Fig. 2. Normalized signals obtained after extractions with various MEPS phases (C8, C18 and M1). Extractions performed with 5 × 50 μl of water samples; concentrations 1.5 μg/ml except for BPA and ATZ 0.75 μg/ml.

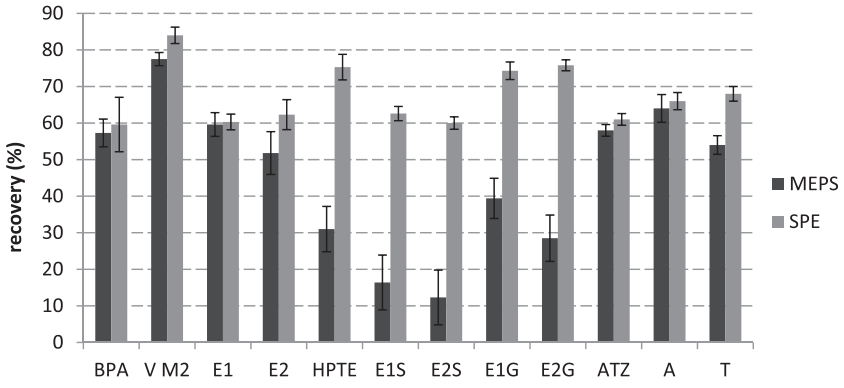

Fig. 3. Recoveries with the MEPS and SPE method, respectively, expressed as percentage of the 100%-recovery spiking.

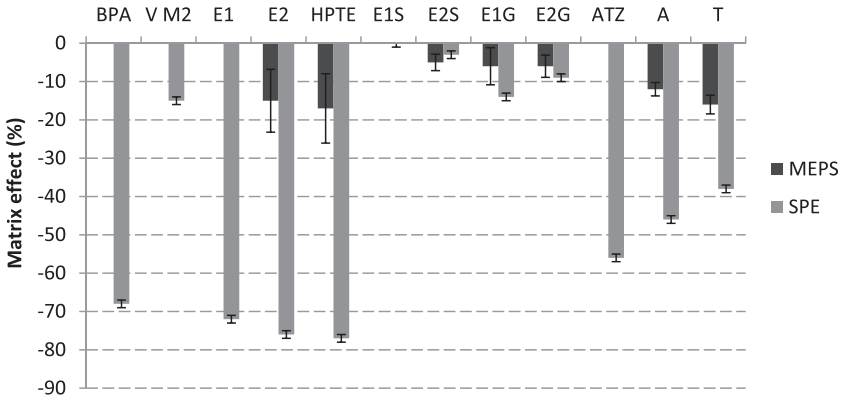

Fig. 4. Drop in the signal with MEPS and SPE method, respectively, in presence of serum matrix expressed as percentage of the spiked water extracts.

frequently observed during LC–MS analyses, influences the ionisation of the analytes, as matrix compounds can be eluted at the same retention time. Fig. 4 shows the drop in the signal in the presence of matrix extract.

It can be observed that matrix effects were dependent on the nature of the target compound. Indeed, the conjugated estrogens were much less subject to the effects than other substances. In the case of E1S it is worth noting that no effect was observed.

It can be also noted that matrix effects were dependent of the sample preparation method. Overall, the effects are much lower after an extraction method by MEPS. Only 7 substances underwent suppression of signal in the MEPS extracts. No significant decrease was observed with BPA, E1, V M2, E1-S and ATZ. On the other hand, all (except E1S) were adversely affected with the protocol based on SPE. The matrix effects are lower than 15% with MEPS but can reach 75% with SPE. The method based on MEPS guaranteed good

clean-up of the matrix. On the other hand, the polymeric phase contained in the SPE cartridge is composed of two monomers allowing the extraction of a wide range of acidic, basic, and neutral interfering compounds from the serum matrix.

In spite of the benefits over matrix effects, these results led us to choose the SPE as the extraction method even though MEPS is a solvent-saving technique. The recoveries obtained by MEPS on conjugated forms were weak. The final 20 × concentration with SPE allows lower methodological limits.

It is worth noting that the compounds exhibit various physico-chemical properties. For example glucuronides are more lipophilic than their parent compound or the structure of atrazine contains amine groups that behave very differently than the alcohols in steroids. Despite these differences, the SPE method was effective at recovering all the target substances.

3.5. LOD–LOQ

As recommended in ICH guideline, the determination of the signal-to-noise ratio was performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio (S/N) is 10:1. The LODs and LOQs were first evaluated by injecting the extracts obtained from spiked serum samples and calculating the matching S/N. LOD and LOQ were reached at S/N = 3 and S/N = 10, respectively. The estimated LOQ were then validated by 10 repetitive injections of extracted animal serum containing the target compounds at the corresponding concentrations. The LOQs were validated if the RSDs of these repetitions were less than 20%.

Table 3 presents the validated values obtained on coupled devices Agilent LC 1200–3200 QTRAP device for both the compounds ionised in positive and negative modes. We noticed better LODs and LOQs for A, T and ATZ than for the estrogenic compounds. Indeed MLQs of A, T and ATZ are below 0.5 ng/ml whereas they are greater than 5 ng/ml for E, EG and HPTE. Afterwards, we noticed better LODs and LOQs of A and T in female sera in the positive mode. This finding could be explained by a lower background noise around the retention times of these molecules (from 5.5 to 7 min). The baseline was less intense, and the chromatograms presented fewer interferences.

The liquid–liquid extraction is still widely used for the analysis of steroids in biological matrices, as evidenced by a recent review [9]. The SPE technique has however been used for analysis of T or A in serum, followed by analysis by UPLC–MS/MS. LOQ of

Table 3
Limits of detection and quantification on the coupled device Agilent LC 1200–3200 QTRAP or Agilent LC 1290–5500 QTRAP, in serum.

		A	T	E1	E2	E1S	E2S	E1G	E2G	BPA	V M2	ATZ	HPTE
LOD (ng/ml)	LC 1200–3200 QTRAP	0.11	0.02	1	2.3	0.033	0.033	1.7	2.7	1.5	0.008	0.037	4
	LC 1290–5500 QTRAP	–	–	0.0084	0.2	0.001	0.0029	0.0037	0.0122	0.093	0.0076	–	0.198
LOQ (ng/ml)	LC 1200–3200 QTRAP	0.41	0.11	1.7	6.9	0.098	0.098	3.05	10.2	3.2	0.075	0.11	19.9
	LC 1290–5500 QTRAP	–	–	0.0277	0.690	0.0028	0.0098	0.0122	0.0405	0.310	0.0277	–	0.660

Table 4
Validation parameters: recoveries, repeatability, reproducibility and linearity obtained with SPE followed by LC–MS/MS with 3200 QTrap spectrometer.

	Transitions (m/z)		Recoveries	Recovery reproducibility	Repeatability (RSD %)			Reproducibility (RSD %)			Linearity	R^2	Calibration curves
	Prec	Prod	(%)	(RSD %)	Low level	Middle level	High level	Low level	Middle level	High level	ng/ml		
BPA	227	133	76	25	8	8	6	23	9	3	3.2–150.0	0.994	$y = 0.0836 \times +0.0961$
	227	117		26	15	14	8	17	11	9			
V M2	258	160	74	12	18	5	5	20	17	17	0.1–3.5	0.996	$y = 26.06 \times +1.8789$
	258	35		12	20	7	5	24	17	17			
HPTE	315	243	78	2	7	9	5	50	25	23	19.9–923.0	0.998	$y = 0.0054 \times +0.0188$
	315	279		6	9	7	7	65	28	26			
E1	269	145	72	7	9	3	3	6	2	1	1.7–77.8	0.999	$y = 0.1679 \times - 0.0298$
	269	159		7	15	2	4	6	2	2			
E2	271	145	82	13	15	7	9	25	3	10	6.9–320.0	0.990	$y = 0.1456 \times +0.529$
	271	183		11	17	5	8	23	3	9			
E1S	349	269	68	21	14	15	7	22	9	7	0.1–4.6	0.996	$y = 0.1755 \times +0.008$
	349	145		25	9	16	7	30	10	8			
E2S	351	271	71	27	16	13	9	31	9	3	0.1–4.6	0.997	$y = 0.2823 \times +0.002$
	351	80		23	20	14	13	22	9	4			
E1G	445	113	79	19	5	5	10	54	12	7	3.1–142.0	0.998	$y = 0.0225 \times +0.0251$
	445	269		10	5	5	10	32	6	5			
E2G	447	113	65	19	1	6	9	13	7	13	10.2–472.0	0.998	$y = 0.0196 \times +0.1039$
	447	271		4	29	5	6	8	14	18			
ATZ	216	174	72	13	3	6	7	6	1	3	0.1–5.0	0.999	$y = 0.2153 \times +0.0003$
	216	104		15	5	4	7	6	1	2			
A	287	97	74	12	10	4	5	8	4	4	0.4–19.0	0.996	$y = 0.1673 \times +0.0358$
	287	109		13	10	4	5	10	5	3			
T	289	97	90	10	10	3	4	27	12	7	0.1–5.0	0.994	$y = 0.1717 \times +0.0121$
	289	109		10	9	1	2	27	14	9			

0.3 ng/mL were thus obtained, with a Quattro Premier/XE triple quadrupole spectrometer [33]. These results are comparable to those we obtained by the validated method, while our method covers a wider range of substances.

In order to increase the sensitivity of the compounds ionised in negative mode, their analysis were performed by Agilent LC 1290–5500 QTRAP device. The results appear in Table 3. As expected, the sensitivity with the 5500 QTRAP was better than the sensitivity with the 3200 QTRAP. The gain of LODs of E2, E1-S and E2-S was by a factor of 10 (11.5, 11.4 and 9, respectively). The LODs were lower by a factor 460 for E1-G, 225 for E2-G, 120 for E1, 20 for HPTE and 16 for BPA, when using 5500QTRAP instead of 3200QTRAP. These gain differences can be explained by the differences in molecule structure, the differences in ionisation and the matrix effect. Only few works dealt with the quantitative analysis of both conjugated and free forms of estrogens in biological matrices. Sirkku et al. [34] developed a sensitive method for the analysis of a wide range of steroids and their glucuronide conjugates. Their LOD were comprised between 0.01 and 5 ng/mL by using an API 3000 triple quadrupole instrument (i.e., equivalent to 3200 QTRAP in term of performance). Their LODs were equivalent to or less sensitive than our validated method.

3.6. Validation

The crucial step of validation involves determining whether the method is consistent with the ICH recommendations and with its application scope.

Recoveries (Table 4) varied between 65% and 90%, with an average of 75%. These results are comparable with previous studies dealing with the analysis of androgen steroids in urine sample [35] or estrogen glucuronides in human serum [36]. The minimum recoveries were 65% and 68% and were obtained for two conjugated hormones (E2G and E1S, respectively). In these cases, even if recoveries values were less than the limit imposed by the standard ICH, the protocol developed for the extraction of E2G and E1S was nevertheless validated since RSDs were less than 20%.

The repeatability of the method, expressed as RSD percentages depended on the compound and the concentration level (Table 4). Thus, for the middle and high levels, RSD values varied between 3% and 15% regarding the quantitative transition. The highest values were obtained for the sulfate conjugates (15% and 13% for E1S and E2S, respectively). RSD values for other compounds did not exceed 10% for the quantitative transition. On the other hand, RSD values were slightly higher for the low concentration level.

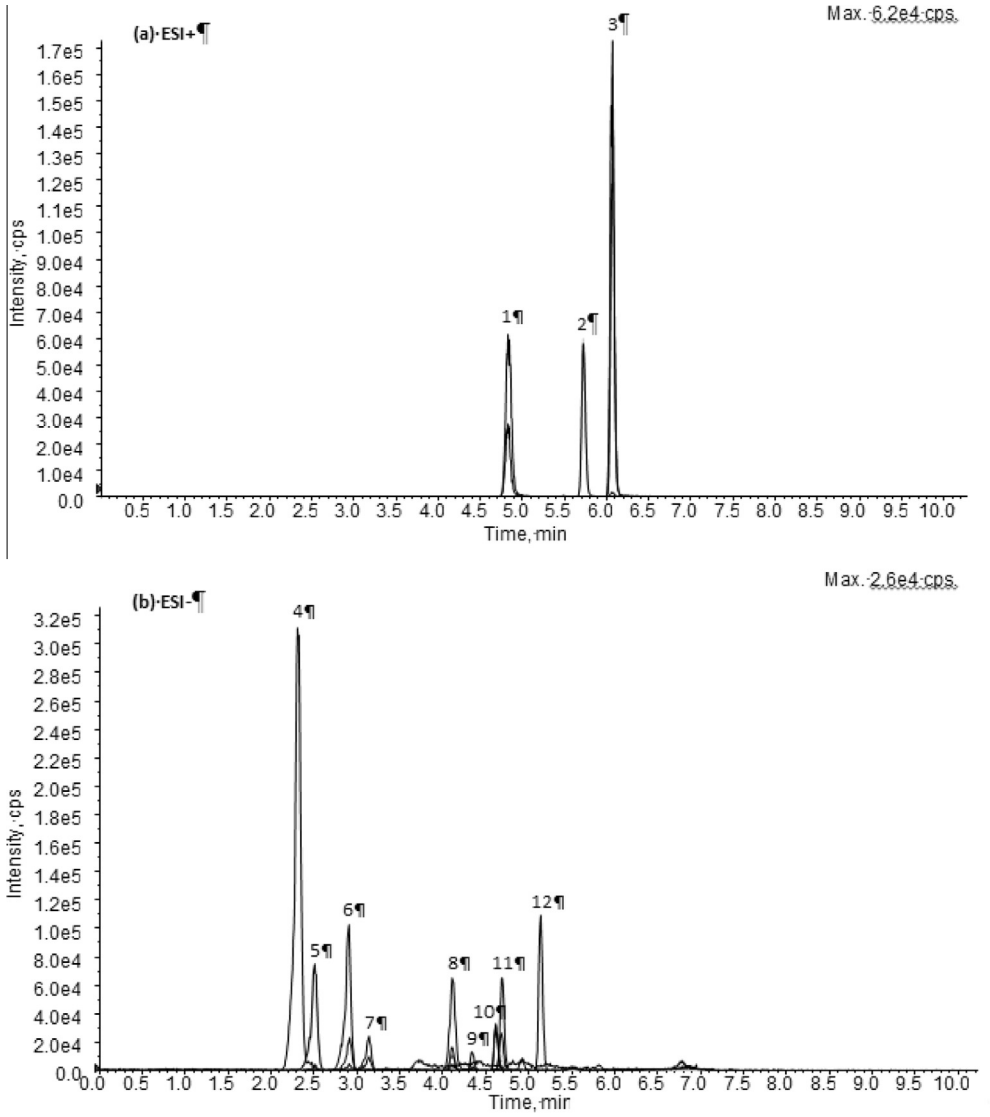

Fig. 5. Total ion chromatograms of a rat serum spiked at a concentration corresponding to 3 × LOQ of each of the target compound then submitted to the whole protocol based on SPE followed by LC–MS/MS. (a) Positive ionisation mode and (b) negative ionisation mode. (1) ATZ, (2) T, (3) A, (4) E2G, (5) E1G, (6) E2S, (7) E1S, (8) BPA, (9) E2, (10) HPTE, (11) E1, (12) VM2.

Indeed, 6 of them were comprised between 10% and 18%. In conclusion, with all the RSD values inferior to 20% the criterion of repeatability was validated regarding ICH guidelines.

The reproducibility was evaluated in the same way as the repeatability (Table 4). The RSD values were also higher for the low concentration level. For the low and middle concentration levels, only the RSD of HPTE exceeded 20%.

To control the repeatability and reproducibility for target molecules, one performance control calibration curve in the reconstitution solvent was performed at each batch. The performance control of the whole process (including sample preparation, chromatographic separation and detection) was effectuated by the presence of the deuterated internal standards.

4. Application to samples from an *in vivo* study

As an example of the suitability of the method, total ion chromatograms corresponding to a young male serum pool spiked at the concentration corresponding to 3 × LOQ of each of the target compounds, then extracted and analysed by the developed method are presented Fig. 5a and b. The validated methodology was applied to serum samples of an *in vivo* study. Both male and female adult rats were involved in this study. The aim was to determine the potential mechanisms of hormone balance disruption following EDC treatments, focusing on hormone production and availability. Doses close to LOAEL (Low Observable Adverse Effect Level) of interest were used. This choice is based on the available literature, reporting effects on hormonal balance and reproductive function. We identified LOAEL of interest for subacute or subchronic oral exposures made during previous studies that were the basis of reflection for the development of this *in vivo* study. Indeed, insofar as this experiment was to characterise the deregulation of hormonal balances in connection with the impairment of fertility, a dose causing effects on these two parameters had to be selected. For ATZ, the LOAEL range from 75 mg/kg [37] to 300 mg/kg [38]. For BPA, LOAEL of alterations on sperm parameters are observed from 0.2 μg/kg [39] to more than 200 mg/kg [40] while the effects on male hormones [41] or on the estrous cycle are reported at 1000 mg/kg [42]. For MCX, LOAEL from 50 mg/kg [43] to more than 500 mg/kg [44] are listed. Finally, LOAEL for VCZ are around 100 mg/kg [45] to 200 mg/kg [46].

On average, in this study, male and female rats weighed 300 and 250 g, respectively, and individual 2-ml serum sample were collected. Consequently, males received daily doses of 60 mg of MXC, ATZ or BPA or 30 mg of VCZ, while females received 50 mg of MXC, ATZ or BPA or 25 mg of VCZ.

Table 5 presents the average concentrations of EDCs in the serum of male and female rats, expressed as the mean ± SEM (standard error of the mean). This table shows a gender difference on the cycle of absorption/elimination. ATZ and V M2 concentrations were higher in female serum than male serum. On the other hand, HPTE and BPA concentrations were similar in males and females. It also shows elevated standard errors of the mean (SEM) as 42.7 ± 38.0 ng/ml of atrazine in female serum, also highlighting an inter-individual variability.

The developed method permitted also a visualisation of the effects of the target endocrine disruptors on estrogen to androgen ratios (Fig. 6). ATZ treatment increased the E2 to T ratio in female serum; VCZ induced a low E2 to T ratio in male serum and low E1 to A ratios in both male and female serums. MXC and BPA treatments increased the E2 to T ratio in female serum. Two different profiles were thus put in evidence: an elevated estrogen-to-androgen ratio following ATZ, MXC and BPA treatment but a decreased estrogen-to-androgen ratio in animals treated with VCZ.

Finally, the method permitted to evaluate free to conjugated hormone ratios (Fig. 7) following ATZ, BPA, MXC or VCZ treatment. A decrease in the E2S to E2 ratio in males treated with ATZ and MXC as well as female treated with VCZ, MXC and BPA was put in evidence. E2G to E2 ratio diminished following male and female treatment with MXC and BPA but increased with ATZ treatment in females. In males, the E1S to E1 ratio was elevated following ATZ treatment but decreased following VCZ, MXC and BPA treatment. E1G to E1 ratio was reduced in males treated with BPA.

Of great interest is the simultaneous measurement of androgenic, estrogenic, free and conjugated steroids. Indeed, it allowed differentiation of the effects on hormone production by the enzyme aromatase (which catalyses the irreversible conversion of androgens to estrogens) from effects on steroid conjugation/deconjugation processes. For example, both ATZ and MXC, classified as estrogenic compounds, elevated estrogens. However, estrogen elevation is likely to be due to aromatase increase activity in ATZ-treated animals (where an elevated estrogen to androgen ratios is described, Fig. 6). It is more likely to be secondary to increased deconjugation or decreased conjugation of estrogens in MXC-treated rats (Fig. 7).

Table 5
Serum dosimetry expressed in ng/ml in male and female rats treated with ATZ 200 mg/kg, VCZ 100 mg/kg, MXC 200 mg/kg, or BPA 200 mg/kg daily for two weeks.

	BPA	ATZ	V M2	HPTE
Males	59.9 ± 26.2	7.3 ± 5.8	9.3 ± 5.3	44.1 ± 23.5
Females	23.7 ± 15.2	42.7 ± 38.0	19.6 ± 8.4	29.7 ± 12.2

Values are mean ± SEM for 10 animals.

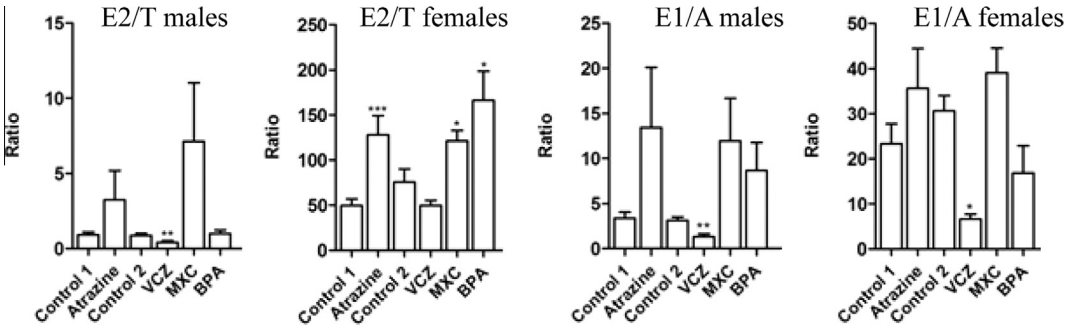

Fig. 6. Estrogens to androgens ratios in serum of males and females treated with atrazine, vinclozolin, methoxychlor, bisphenol A daily for two weeks The graphs represent estradiol to testosterone and estrone to androstenedione ratios in serum in male (A) and female (B) adult rats treated or not (control) with atrazine 200 mg/kg, vinclozolin 100 mg/kg, methoxychlor 200 mg/kg, bisphenol A 200 mg/kg daily for two weeks. Values are mean ± SEM of 10 animals per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus corresponding control.

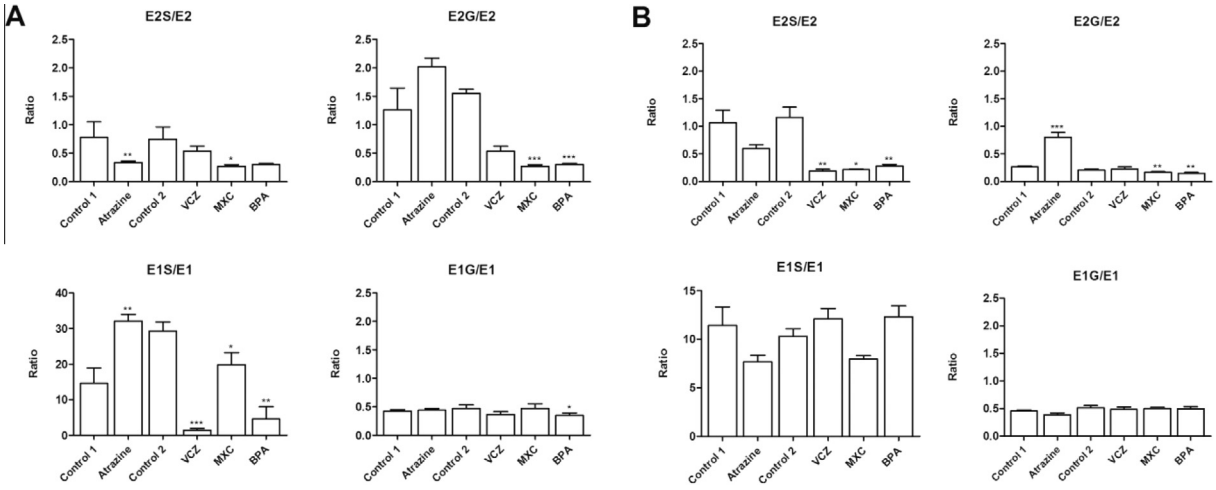

Fig. 7. Conjugated to free estrogen ratios in serum of males and females treated with ATZ, VCZ, MCX, BPA daily for two weeks The graphs represent serum estradiol-sulfate/glucuronide to estradiol, and estrone-sulfate/glucuronide to estrone ratios in male (A) and female (B) adult rats treated or not (control) with atrazine 200 mg/kg, vinclozolin 100 mg/kg, methoxychlor 200 mg/kg, bisphenol A 200 mg/kg daily for two weeks. Values are mean ± SEM of 10 animals per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus corresponding control.

5. Conclusions

A simple, quick, selective and efficient method was implemented for the determination of steroid hormones (free and conjugated forms) and endocrine disruptors in rat serum. Even if MEPS technique is solvent-saving (a "green" method as widely named), it is not appropriate for conjugated hormones. New polymeric phase BINs need to be developed. Therefore the SPE technique was chosen for sample preparation. This step is simple because the protocol is constituted of one solid-phase extraction and is rapid because the extraction is performed in less than 10 min. Ten samples can be extracted in one hour using only one device.

The LC–MS/MS method analysis, which is highly sensitive, accurate, precise and specific, allows the simultaneous detection and quantification of two classes of molecules in small serum samples (a few mLs of sample). The multi-residue method was validated according to the criteria of the standard ICH, and as a consequence, it has been applied to 260 serum samples providing interesting information about toxicological patterns of EDCs. Due to its great adaptability, this procedure could be applied to other biologic fluids or other species (such as human urine).

References

[1] European Commission, SEC, 2007, 1635, available at <http://ec.europa.eu/environment/endocrine/documents/index_en.htm>.
[2] World Health Organization, 2002, Global assessment of the state-of-the-science of endocrine disruptors, WHO/PCS/EDC/02.2, available at <http://www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en/>.
[3] E. Swedenborg, J.I. Rüegg, S. Makela, I. Pongratz, Endocrine disruptive chemicals: mechanisms of action and involvement in metabolic disorders, J. Mol. Endocrinol. 43 (2009) 1–10.
[4] V.R. Agarwal, S.E. Bulun, M. Leitch, R. Rohrich, E.R. Simpson, Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients, J. Clin. Endocrinol. Metab. 81 (1996) 3843–3849.
[5] A. Kamat, M.M. Hinshelwood, B.A. Murry, C.R. Mendelson, Mechanisms in tissue-specific regulation of estrogen biosynthesis in human, Trends Endocrinol. Metab. 13 (2002) 122–128.
[6] L.E. Gray Jr., V. Wilson, N. Noriega, C. Lambright, J. Furr, T.E. Stoker, S.C. Laws, J. Goldman, R.L. Cooper, P.M.D. Foster, Use of the laboratory rat as a model in endocrine disruptor screening and testing, ILAR J. 45 (2004) 425–437.
[7] W.S. Stokes, Selecting appropriate animal models and experimental designs for endocrine disruptor research and testing studies, ILAR J. 45 (2004) 387–393.
[8] O. Nozaki, Steroid analysis for medical diagnosis, J. Chromatogr. A 935 (2001) 267–278.
[9] F. Gosetti, E. Mazzucco, M.C. Gennaro, E. Marengo, Ultra high performance liquid chromatography tandem mass spectrometry determination and profiling of prohibited steroids in human biological matrices. A review, J. Chromatogr. B 927 (2013) 22–36.
[10] L. Nováková, H. Vlcková, A review of current trends and advances in modern bio-analytical methods: chromatography and sample preparation, Anal. Chim. Acta 656 (2009) 8–35.
[11] D.T. Harwood, D.J. Handelsman, Development and validation of a sensitive liquid chromatography–tandem mass spectrometry assay to simultaneously measure androgens and estrogens in serum without derivatization, Clin. Chim. Acta 409 (2009) 78–84.
[12] F. Lafay, E. Vulliet, M.-M. Flament-Waton, Contribution of microextraction in packed sorbent for the analysis of cotinine in human urine by GC–MS, Anal. Bioanal. Chem. 396 (2011) 937–941.
[13] M. Abdel-Rehim, Recent advances in microextraction by packed sorbent for bioanalysis, J. Chromatogr. A 1217 (2011) 2569–2580.
[14] J. Aufartová, C. Mahugo-Santana, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, L. Nováková, P. Solich, Determination of steroid hormones in biological and environmental samples using green microextraction techniques: an overview, Anal. Chim. Acta 704 (2011) 33–46.
[15] A. Palenikova, S. Hrouzkova, Microextraction in packed syringe: solvent-minimized sample preparation technique, Monatsch. Chem. 145 (2014) 537–549.
[16] M. Abdel-Rehim, AstraZeneca application "syringe for solid phase microextraction", Current Patents Gazette, week 0310, WO 03019149, 2003, p. 77.
[17] F. Labrie, A. Bélanger, P. Bélanger, R. Bérubé, C. Martel, L. Cusan, J. Gomez, B. Candas, I. Castiel, V. Chaussade, C. Deloche, J. Leclaire, Androgen glucuronides, instead of testosterone, as the new markers of androgenic activity in women, J. Steroid Biochem. Mol. Biol. 99 (2006) 182–188.
[18] X. Xu, J.M. Roman, H.J. Issaq, L.K. Keefer, T.D. Veenstra, R.G. Ziegler, Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography–tandem mass spectrometry, Anal. Chem. 79 (2007) 7813–7821.
[19] F. Qin, Y.-Y. Zhao, M.B. Sawyer, X.-F. Li, Hydrophilic interaction liquid chromatography–tandem mass spectrometry determination of estrogen conjugates in human urine, Anal. Chem. 80 (2008) 3404–3411.
[20] F. Qin, Y.Y. Zhao, M.B. Sawyer, X.-F. Li, Column-switching reversed phase–hydrophilic interaction liquid chromatography/tandem mass spectrometry method for determination of free estrogens and their conjugates in river water, Anal. Chim. Acta 627 (2008) 91–98.
[21] P. Caron, E. Audet-Walsh, J. Leì Pine, A. Beì Langer, C. Guillemette, Profiling endogenous serum estrogen and estrogen-glucuronides by liquid chromatography–tandem mass spectrometry, Anal. Chem. 81 (2009) 10143–10148.
[22] E. Strahm, I. Kohler, S. Rudaz, S. Martel, P.-A. Carrupt, J.-L. Veuthey, M. Saugy, C. Saudan, Isolation and quantification by high-performance liquid chromatography–ion-trap mass spectrometry of androgen sulfoconjugates in human urine, J. Chromatogr. A 1196–1197 (2008) 153–160.
[23] F. Giton, P. Caron, R. Bérubé, A. Bélanger, O. Barbier, J. Fiet, Plasma estrone sulfate assay in men: comparison of radioimmunoassay, mass spectrometry coupled to gas chromatography (GC–MS), and liquid chromatography–tandem mass spectrometry (LC–MS/MS), Clin. Chim. Acta 411 (2010) 1208–1213.
[24] H. Hiroi, O. Tsutsumi, M. Momoeda, Y. Takai, Y. Osuga, Y. Taketani, Differential interactions of bisphenol A and 17beta-estradiol with estrogen receptor alpha (ERalpha) and ERbeta, Endocr. J. 46 (1999) 773–778.
[25] S.C. Hewitt, K.S. Korach, Estrogenic activity of bisphenol A and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) demonstrated in mouse uterine gene profiles, Environ. Health Perspect. 119 (2011) 63–70.

[26] F. Orton, E. Rosivatz, M. Scholze, A. Kortenkamp, Widely used pesticides with previously unknown endocrine activity revealed as in vitro antiandrogens, Environ. Health Perspect. 119 (2011) 794–800.
[27] T.B. Hayes, L.L. Anderson, V.R. Beasley, S.R. de Solla, T. Iguchi, H. Ingraham, P. Kestemont, J. Kniewald, Z. Kniewald, V.S. Langlois, E.H. Luque, K.A. McCoy, M. Muñoz-de-Toro, T. Oka, C.A. Oliveira, F. Orton, S. Ruby, M. Suzawa, L.E. Tavera-Mendoza, V.L. Trudeau, A.B. Victor-Costa, E. Willingham, Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes, J. Steroid Biochem. Mol. Biol. 127 (2011) 64–73.
[28] K.W. Gaido, S.C. Maness, D.P. McDonnell, S.S. Dehal, D. Kupfer, S. Safe, Interaction of methoxychlor and related compounds with estrogen receptor alpha and beta, and androgen receptor: structure-activity studies, Mol. Pharmacol. 58 (2000) 852–858.
[29] J. Bursztyka, L. Debrauwer, E. Perdu, I. Jouanin, J.-P. Jaeg, J.-P. Cravedi, Biotransformation of vinclozolin in rat precision-cut liver slices: comparison with in vivo metabolic pattern, J. Agric. Food Chem. 56 (2008) 4832–4839.
[30] E. Vulliet, L. Wiest, R. Baudot, M.-F. Grenier-Loustalot, Multi-residue analysis of steroids at sub ng/L levels in surface and ground-waters using liquid chromatography coupled to tandem mass spectrometry, J. Chromatogr. A 1210 (2008) 84–91.
[31] International Conference on Harmonisation (ICH), Harmonised tripartite guideline validation of analytical procedures: text and methodology Q2(R1) (2005).
[32] M. Abdel-Rehim, Microextraction by packed sorbent (MEPS): a tutorial, Anal. Chim. Acta 701 (2011) 119–128.
[33] E. Kulle, F.G. Riepe, D. Melchior, O. Hiort, P.M. Holterhus, A novel ultrapressure liquid chromatography tandem mass spectrometry method for the simultaneous determination of androstenedione, testosterone, and dihydrotestosterone in pediatric blood samples: age- and sex-specific reference data, J. Clin. Endocrinol. Metab. 95 (2010) 2399–2409.
[34] E. Sirkku, S.E. Jäntti, M. Hartonen, M. Hilvo, H. Nygren, T. Hyötyläinen, R.A. Ketola, R. Kostiainen, Steroid and steroid glucuronide profiles in urine during pregnancy determined by liquid chromatography–electrospray ionization–tandem mass spectrometry, Anal. Chim. Acta 802 (2013) 56–66.
[35] F. Buiarelli, F. Cocciolo, M. Merolle, B. Neri, A. Terracciano, Development of a liquid chromatography–tandem mass spectrometry method for the identification of natural androgen steroids and their conjugates in urine samples, Anal. Chim. Acta 526 (2011) 113–120.
[36] P. Caron, E. Audet-Walsh, J. Lépine, A. Bélanger, C. Guillemette, Profiling endogenous serum estrogen and estrogen-glucuronides by liquid chromatography–tandem mass spectrometry, Anal. Chem. 81 (2009) 10143–10148.
[37] R.L. Cooper, T.E. Stoker, J.M. Goldman, M.B. Parrish, L. Tyrey, Effect of atrazine on ovarian function in the rat, Reprod. Toxicol. 10 (1996) 257–264.
[38] H. Shibayama, T. Kotera, Y. Shinoda, T. Hanada, T. Kajihara, M. Ueda, H. Tamura, S. Ishibashi, Y. Yamashita, S. Ochi, Collaborative work on evaluation of ovarian toxicity. 14) Two- or four-week repeated-dose studies and fertility study of atrazine in female rats, J. Toxicol. Sci. 34 (Suppl. 1) (2009) SP91–SP99.
[39] K.C. Chitra, C. Latchoumycandane, P.P. Mathur, Induction of oxidative stress by bisphenol A in the epididymal sperm of rats, Toxicology 185 (2003) 119–127.
[40] J. Ashby, H. Tinwell, P.A. Lefevre, R. Joiner, J. Haseman, The effect on sperm production in adult Sprague–Dawley rats exposed by gavage to bisphenol A between postnatal days 91–97, Toxicol. Sci. 74 (2003) 129–138.
[41] D. Nakamura, Y. Yanagiba, Z.W. Duan, Y. Ito, A. Okamura, N. Asaeda, Y. Tagawa, C.M. Li, K. Taya, S.Y. Zhang, Bisphenol A may cause testosterone reduction by adversely affecting both testis and pituitary systems similar to estradiol, Toxicol. Lett. 194 (2010) 16–25.
[42] K. Yamasaki, M. Sawaki, S. Noda, N. Imatanaka, M. Takatsuki, Subacute oral toxicity study of ethynylestradiol and bisphenol A, based on the draft protocol for the "Enhanced OECD test guideline n°407", Arch. Toxicol. 76 (2002) 64–74.
[43] C. Latchoumycandane, K.C. Chitra, P.P. Mathur, The effect of methoxychlor on the epididymal antioxidant system of adult rats, Reprod. Toxicol. 16 (2012) 161–172.
[44] K. Okazaki, S. Okazaki, S. Nishimura, H. Nakamura, Y. Kitamura, K. Hatayama, A. Nakamura, T. Tsuda, T. Katsumata, A. Nishikawa, M. Hirose, A repeated 28-day oral dose toxicity study of methoxychlor in rats, based on the "Enhanced OECD test guideline 407" for screening endocrine-disrupting chemicals, Arch. Toxicol. 75 (2001) 513–521.
[45] K. Kubota, S. Ohsako, S. Kurosawa, K. Takeda, W. Qing, M. Sakaue, T. Kawakami, R. Ishimura, C. Tohyama, Effects of vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat, J. Reprod. Dev. 49 (2003) 403–412.
[46] J.H. Shin, H.J. Moon, T.S. Kim, I.H. Kang, H.Y. Ki, K.S. Choi, S.Y. Han, Repeated 28-day oral toxicity study of vinclozolin in rats based on the draft protocol for the "Enhanced OECD test guideline n°407" to detect endocrine effect, Arch. Toxicol. 80 (2006) 547–554.