Highlights

- A new fabric phase sorptive extraction combined with UHPLC-MS/MS is proposed.
- The method is optimized and validated to determine six androgens and four progestogens.
- All extraction and desorption variables have been optimized.
- Minimum quantities of sample and organic solvents and short extraction times are used.
- Water and urine samples have been successfully analyzed using the proposed method.

DETERMINATION OF ANDROGENS AND PROGESTOGENS IN ENVIRONMENTAL AND BIOLOGICAL SAMPLES USING FABRIC PHASE SORPTIVE EXTRACTION COUPLED TO ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

Androgens and progestogens are two important groups of endocrine disrupting compounds (EDCs) which are implicated to produce severe detrimental impact over aquatic biota, even at very low concentrations of ng·L^{-1}. For this reason, one of the major challenges to analytical chemists is the development of sensitive and selective extraction processes which allow the rapid and green determination of these emerging pollutants at low concentrations in environmental samples. Fabric phase sorptive extraction is a new, highly sensitive, efficient and solvent minimized technique which combine the advantages of sol–gel derived microextraction sorbents and the rich surface chemistry of cellulose fabric substrate. This process has several advantages such as minimum usage of organic solvents, short extraction times, small sample volumes and high analyte preconcentration factors. In this study, an extraction method based on sorptive fabric phase coupled to ultra-high-performance liquid chromatography tandem mass spectrometry detection (FPSE-UHPLC-MS/MS) has been developed for the determination of four progestogens and six androgens in environmental and biological samples. All the parameters involved in the extraction, such as sample volume, extraction and desorption times, desorption solvent volume and sample pH values have been optimized. The developed method provides satisfactory limits of detection (between 1.7 and 264 ng·L^{-1}), good recoveries and low relative standard deviations (below 10% in tap and osmosis water and below 20% in wastewater and urine). Subsequently, the method was used to analyse tap water, wastewater treated with different processing technologies and urine samples. The concentrations of the detected hormones ranged from 28.3 to 227.3 ng·L^{-1} in water samples and from 1.1 to 3.7 µg·L^{-1} in urine samples.

Keywords

Endocrine disrupting compounds, Androgens, Progestogens, Ultra High Performance Liquid Chromatography, Wastewater, Urine

1. Introduction

Steroid sex hormones are biologically active compounds involved in almost all vital physiological functions of the body *via* genomic and non-genomic effects. Based on structural differences and affinities, steroid hormones can be divided into five subclasses: estrogens, androgens, progestogens, glucocorticoids and mineralcorticoids.

Various diseases are related to disorders of the homeostasis of steroid hormones; the mechanisms by which these compounds mediate their biological effects provides opportunities for pharmacological interventions in various clinical conditions [1]. Their quantification in body fluids (e.g. urine) will help in understanding the individual biochemical responses to the disease and its progression, and in achieving better personalized medicine [2]. Nowadays, natural and synthetic steroid hormones find a wide use in both human and veterinary medicine [3].

Steroid hormones are excreted by humans and animals and subsequently reach the surface waters due to direct discharge and their incomplete removal in wastewater treatment plants (WWTPs) which, together with hospital effluents, have been reported to be the main source of contamination of the aquatic environments [4,5].

Since the first evidence of feminization of fish exposed to WWTP effluents [6], a remarkable effort has been waged to assess the presence of oestrogens and estrogenic endocrine disrupting compounds in water [7]. However, more recently it has been demonstrated that environmental exposure of aquatic organism to androgens and progestogens may also cause adverse effects even at very low concentration levels (low $ng \cdot L^{-1}$) [8–10]; for this reason these compounds have been designated as endocrine disrupting chemicals (EDCs), which represent nowadays a topic of high concern for national and international organizations and regulatory agencies committed to public and environmental health.

The development of fast, reliable, sensitive and green analytical methods for the determination of androgens and progestogens in water matrixes is of crucial importance for the assessment of the concentration levels of these compounds and their related ecological risk.

Chromatographic techniques represent today the reference analytical methods for the analysis of steroid hormones in biological and environmental samples, and numerous analytical procedures have been developed based on gas chromatography (GC) and liquid chromatography (LC) coupled to single stage (MS) or tandem mass (MS/MS) detection [3]. However, GC analysis of steroid hormones is time consuming and labour intensive since, due to their molecular weights and reduced volatility, a derivatization step is required [11–14]. Thanks to its selectivity, sensitivity, analytical throughput and compatibility with the physico-chemical properties of steroid hormones, LC-MS(/MS) has been extensively applied to the analysis of these compounds in a wide range of liquid and solid matrices [15–25].

Due to the low concentration levels of steroid hormones in surface water and wastewater, together with the complexity of both environmental and biological matrices in which these compounds are dispersed, a preconcentration and clean-up step are usually carried out [3,26]. The most popular sample preparation technique is solid-phase extraction, and has widely employed for steroid hormones analysis of water samples by several authors, both manually [16,19–21,24,27] and automatically [15,17,25].

Miniaturization of extraction techniques is the main trend with this type of compounds and new sorptive extraction methods as solid-phase microextraction or stir bar sorptive extraction has been investigated by many authors [18,28,29] in order to achieve a reduction in the volume of both sample and organic solvent employed during the whole sample preparation procedure, as well as to move forward to a greener chemistry. Recently, Kabir and Furton developed a novel extraction medium, known as fabric phase sorptive extraction (FPSE), which exploits the advantages of the sol-gel chemistry and the intrinsic high surface area of cellulosic materials, overcoming some major limitations of the current sol-gel SPME formats, namely low sample capacity and longer sample preparation time [30]. Sol-gel coating technology overcame some of the limitations of the traditional coatings, especially thanks to its low costs, molecular-scale uniformity and chemical-bonding to the substrate [30].

The aim of this study was to develop a simple, fast and sensitive analytical method for the quantification of natural and synthetic androgens and progestogens by coupling sol-gel

poly(tetrahydrofuran) coated FPSE to UHPLC-MS/MS analysis, so as to exploit the advantages of the novel sample preparation technique together with the recent improvements of the liquid chromatography instrumentation and column technology. The key parameters that affect the extraction efficiency (i.e. extraction time, desorption time and ionic strength) were reliably optimized by means of a 2^3 followed by a 3^2 factorial experimental design conducted on standard solutions. Other variables, such as pH and sample volume, were also investigated. The applicability of the method to the analysis of environmental and biological samples was verified on wastewater treated with different techniques from wastewater treatment plants of Gran Canaria (Spain), as well as on urine samples.

2. Materials and methods

2.2 Material, solvents and reagents

Ultrapure water used was provided by a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol, LC-MS methanol, and LC-MS water as well as the ammonia to adjust the pH of the mobile phase were obtained from Panreac Química (Barcelona, Spain). All of the steroid hormones used (Table 1) were purchased from Sigma–Aldrich (Madrid, Spain). Stock solutions containing 1000 $mg \cdot L^{-1}$ of each analyte were prepared by dissolving the compound in methanol, and the solutions were stored in glass-stoppered bottles at -20°C prior to use. Working standard solutions were prepared daily.

The sorbent coating of the fabrics used in the FPSE was sol-gel poly(tetrahydrofuran). The preparation and characterization of sol-gel poly(tetrahydrofuran) coated FPSE have been described in previous articles [30].

2.3 Sample collection

2.3.1 Environmental water and wastewater samples

Wastewater samples were collected from the secondary and tertiary effluents of a wastewater treatment plant of island of Gran Canaria (Spain) that purifies the water of a high-density population area with an approximate population of 260,000. Another sample was collected from the untreated effluent of the hospitalization area of Las Palmas de Gran Canaria (Spain) and tap water was collected in the university area of Las Palmas de Gran Canaria. All the samples were collected in June of 2015 in 2 L amber glass bottles that were rinsed beforehand with methanol and ultrapure water. After collecting the water samples, they were purified through filtration with fibreglass filters and 0.22 μm membrane filters (Millipore, Ireland), and were stored in the dark at 4°C and extracted within 48 hours.

2.3.2 Urine samples

10 mL of urine samples were obtained from healthy men and women. Before the extraction procedure, the urine sample was centrifuged at 3500 r.p.m. for 10 minutes and the supernatant was collected. 2 mL of the supernatant aliquots were filtered through PET 0.2 μm syringe filters (Macherey-Nagel, Düren, Germany), diluted 10 times with ultrapure Milli-Q water and degassed on ultra-sonic bath for 10 minutes.

2.4 Instrumentation and chromatographic conditions

An ultra-high performance liquid chromatography system coupled to a triple quadrupole detector (UHPLC-MS/MS) has been used. It consists of an ACQUITY Quaternary Solvent Manager used to load samples as well as to wash and recondition the analytical column, an autosampler capable of injecting volumes up to 25 μL per injection for up to 21 vials, a column manager and a triple quadrupole detector, which were all from Waters (Barcelona, Spain). The detection parameters for each compound are shown in Table 2.

The analytical column was a 50 mm × 2.1 mm, ACQUITY UHPLC BEH Waters C_{18} column with a particle size of 1.7 μm (Waters, Barcelona, Spain) operating at a temperature of 30°C. The sample volume injected was 10 μL, and the analyte separation was carried out using water

with 0.1% (v/v) of ammonia and methanol without additives at a flow rate of 0.3 $mL \cdot min^{-1}$ in gradient mode. The gradient started at an 80:20 (v/v) mixture of water:MeOH, which changed to 40:60 (v/v) in 1.5 minutes and to 25:75 (v/v) in 1.25 minutes more. Then, the gradient changed to 0:100 (v/v) in 1 minute. Finally, it returned to 80:20 in 2.25 minute and stayed at that mixture for calibration for an additional 0.5 minutes. Thus, the chromatographic separation was completed in 6.5 minutes.

2.5 Fabric phase sorptive extraction procedure

Before carrying out the extraction, fabric media was immersed in 2 mL of a mixture of methanol: acetonitrile (50:50, v/v) followed by immersing in 2 mL of ultrapure Milli-Q water for 10 minutes in order to clean and activate the sol-gel coating for the extraction. Subsequently, 10 mL of water and 20 mL of diluted urine samples (spiked with a concentration of 10 and 50 $\mu g \cdot L^{-1}$ of each compound, respectively) were placed in glass vials with a Teflon coated magnetic stirrer. The sol-gel poly(tetrahydrofuran) coated fabric media was submerged into the sample solution and was stirred at 1000 rpm for the optimum extraction time. After that, the extraction media was removed from the vial, submerged into back-extracting solvent to do the elution of the analytes and the eluent was injected into the chromatographic system. To avoid the potential carryover effects, the fabrics were washed by immersing it in 2 mL of methanol for 5 minutes and the methanol used for washing was injected to check the absence of target compounds. Finally, the fabrics were dried for 10 minutes before storage.

3. Results and discussion

3.1 Optimization of the fabric phase sorptive extraction

Several parameters can affect the FPSE such as the sample volume, ionic strength of the aqueous sample matrix, pH of the sample, the extraction and desorption times and the volume

desorption solvent. To study the optimum conditions, an experimental design has been used for the most dependent variables, which are the extraction and desorption time and the ionic strength of the sample. Firstly, a 2^3 fractional factorial experimental design has been used to study the significance of each variable and the correlation/interaction between them. Finally, another 3^2 experimental design was built with the variables possessing the major partial correlation. Once optimized this three variables, different values of sample pH and sample and desorption volumes have been tested in order to find the optimum extraction conditions.

3.1.1 *Extraction and desorption times and sample ionic strength optimization.*

FPSE is strongly affected by extraction and desorption times because these factors are directly related to the distribution coefficients of the compounds which establish the adsorption equilibrium between the FPSE sorptive medium and the sample solution. Moreover, it is important to study the presence of a salt in the sample, because it can affect the extraction equilibrium. It is known that the addition of a salt in equilibrium extraction process can produce an increase of the extraction efficiency in compounds with log $K_{OW} < 3$ [31]. To evaluate these three variables, an experimental design of 2^3 was used, using Statgraphics Plus software 5.1 to do the experimental design, while the statistical analysis was done with IBM SPSS Statistics 19. Two levels and three parameters: extraction time (10 and 30 minutes), ionic strength (0 and 15% (w/v) of NaCl) and desorption time (2 and 10 minutes) were tested, to obtain the influence of each parameter and the interactions among each other. The results showed that longer extraction times were slightly worse than short extraction times, as well as, the addition of salt has a negative influence in the extraction of the analytes from the samples. Regarding the desorption time, the correlations showed (Table 3) that it has a moderate to high negative contribution to the extraction of the analytes. For this reason, the salt of the sample was fixed to 0% of addition of NaCl (w/v). Moreover, the correlations between extraction and desorption times were quite moderate for most compounds, so a new experimental design of 3^2 was built to study the relation between these variables. The levels tested for each parameter were 10, 20 and 30 minutes of extraction and 2, 4 and 6 minutes for desorption. In Figure 1 can be seen response

surfaces of the second experimental design for different compounds which show an extraction efficiency maximum in 20 minutes of extraction and 3 minutes of desorption.

3.1.2 Sample pH optimization.

Four different pH values were tested, one acid pH (pH = 2.1) the pH of the sample (pH = 5.7) and two basic pH values (pH =10.0 and 12.0). The greatest peak area values were achieved at the sample pH (pH = 5.7) and as can be seen in Figure 2, the extractions at pH values which are 5 or more units lower than the pKa values of the compounds are more effective because the extractions are performed with neutral molecules. When the sample pH value is near the pKa values of the target compounds, the efficiency of the extraction decreases. Moreover, Dunnett T3 nonparametric test was used to see if the results of each pH were statically different. The results show that and in most of the cases there are no statistically significant differences between the pH values below the pKa of the compounds.

3.1.3 Sample and desorption volume optimization.

The sample volume and the volume of the desorption solvent used in the FPSE are strongly related with the preconcentration capacity of the technique. For this reason, two sample volumes (10 and 20 mL) and two desorption solvent volumes (1.5 and 0.75 mL) were tested. For the Milli-Q water the back-extraction recoveries were slightly higher using 10 mL of sample than 20 mL. On the other hand, for urine samples no important differences in the back-extraction recoveries were detected between both studied volumes, but the recoveries using 20 mL of sample were slightly higher than 10 mL. Regarding to the desorption solvent volume, the back-extraction recoveries using 1.5 or 0.75 mL of methanol were practically similar, so a volume of 0.75 mL of methanol was established as optimum, for both type of samples because the use of small quantities of organic solvent provide a better preconcentration factor. Figure 3 shows the back-extraction efficiencies of the different compounds studied in Milli-Q water and urine.

In accordance with the obtained results, the optimum conditions for the fabric phase sorptive extraction procedure were as follows: extraction for 20 minutes of the optimum volume of the different samples at a pH of 5.70 and 0% of NaCl, and desorption with 0.75 mL of methanol during 3 minutes. In these conditions, the theoretical preconcentration factor was calculated as 13.3 for the environmental water and wastewater samples and 26.6 for urine samples.

3.2 Analytical parameters and quality control

The linearity, recovery, repeatability, limits of detection and limits of quantification of FPSE method were evaluated in the optimum extraction conditions for each kind of samples. External calibration curves were prepared in the range between 0.5 and 400 $\mu g \cdot L^{-1}$ of each compound. Moreover, two internal standards (testosterone D3 and progesterone D9), at a fixed concentration of 200 $\mu g \cdot L^{-1}$, were added to each calibration level. The linearity was calculated using the relationship between areas and concentrations of compounds and internal standards with excellent correlation coefficients (r^2) higher than 0.997.

The relative recoveries were studied using three samples of wastewater spiked with the target compounds at a concentration level of 10 $\mu g \cdot L^{-1}$ and 50 $\mu g \cdot L^{-1}$ by calculating the ratio between the response of the extracted sample with analytes and the response of post-extracted spiked samples[32]. As seen in Table 4, the higher recoveries were obtained in the tap water samples. For the wastewaters, the recoveries were slightly lower and this can be explained by the presence of different salts and other matrix interferents in the wastewaters, which could reduce the effectiveness of the adsorption of the target compounds.

The repeatability of the method was evaluated intra- and inter-day using a triplicate analysis of each sample. They were spiked with target compounds at a concentration levels of 10 $\mu g \cdot L^{-1}$ and 50 $\mu g \cdot L^{-1}$. Both repeatability values were satisfactory and the relative standard deviations were, in all cases, below 20%.

The method detection and quantification limits (LOD and LOQ) for each compound were

253 calculated from the signal to noise ratio of each individual peak. The LOD was defined as the
254 lowest concentration that gave a signal to noise ratio that was equal to 3. The LOQ was defined
255 as the lowest concentration that gave a signal to noise ratio that was equal to 10. For
256 environmental waters, the LOD values calculated for the target compounds ranged from 1.7 to
257 264 $ng \cdot L^{-1}$, while they were from 8.9 to 132.2 $ng \cdot L^{-1}$ for urine samples. The LOQ values were
258 from 5.7 to 880 $ng \cdot L^{-1}$ for environmental water samples and from 29.7 to 440.7 $ng \cdot L^{-1}$ for urine
259 samples.
260 Furthermore, in analysis with MS/MS and electrospray ionization, the composition of complex
261 matrices, as wastewater or urine, has a great influence in the analytical signal. In this sense, an
262 enhancement or suppression of the signal could be produced by co-eluted compounds which
263 would interfere in the good ionization of the compounds under study. To evaluate this
264 phenomenon, spiked matrix extracts and pure standard solutions have been compared in order to
265 evaluate the possible suppressions or enhancements of the analytical signal. Figure 4 shows
266 that, in wastewater samples, a slightly enhancement of the analytical signal (below 20%) is
267 produced, except for androsterone, which has a signal enhancement of 37.5%. For urine
268 samples, matrix effects have lower values, between –10 and +7% for all the compounds. These
269 low matrix effects show that the developed extraction method has an excellent selectivity and
270 the possible interferences extracted from the samples do not affect the detection of the target
271 analytes.
272 The FPSE-UHPLC-MS/MS developed method resolves the main drawbacks of other analytical
273 methods for the determination of androgens and progestogens in environmental samples. As can
274 be seen in Table 5, some authors use other extraction techniques as bar adsorptive
275 microextraction (BAμE) or stir bar sorptive extraction (SBSE) [18,33] with similar sample
276 volumes than the volume used in this work, but the coupling to a chromatographic system with
277 optical detectors causes higher detection limits than the limits reached in this study. Other
278 works use solid phase extraction in both on-line [15,17,34] and off-line [12,20,35] modes with
279 similar detection limits, nevertheless FPSE method do not need a special device to carry out the

extraction as on-line SPE methods and the sample volumes are 10 to 100 times lower than the off-line SPE methods.

3.3 Analysis of androgens and progestogens in wastewater and urine samples

The optimized method was used for the identification and determination of target hormones in different real samples of wastewater from WWTP, untreated hospital wastewater, tap water and urine. Figure 5 shows the chromatograms of real samples of hospital untreated wastewater and urine, where can be seen the adequate separation and detection of the hormones found and the adequate selectivity of the FPSE method.

Target compounds were detected in the secondary effluent, hospital untreated influent and urine samples. As can be seen in Table 6, in urine samples were detected three natural hormones (progesterone, testosterone and androstenedione) at higher concentrations, in the range of $\mu g \cdot L^{-1}$. In the wastewater samples, the untreated effluent of the hospital showed higher concentrations of progesterone than the secondary treatment samples. Moreover, megestrol acetate, boldenone, testosterone and androstenedione were detected but not quantified, because all of them were detected below the quantification limit except testosterone in secondary treatment samples which was detected at 22.8 $ng \cdot L^{-1}$. No hormones under study were detected either in osmosis treatment samples, or in tap water samples.

4. Conclusions

A new fabric phase sorptive extraction (FPSE) method has been developed and was successfully applied to liquid environmental and biological samples for the determination of a group of ten progestogens and androgens. All the parameters related to FPSE, such as sample volume, desorption solvent volume, extraction and desorption times, impact of salt addition, pH of the sample have been optimized in order to get the better recoveries for all compounds.

The developed FPSE-UHLPC-MS/MS method shows a good selectivity and sensitivity and it offers low detection limits that ranged from 1.7 $ng \cdot L^{-1}$ to 264 $ng \cdot L^{-1}$, which are appropriate in

the analysis of endocrine disrupting compounds in environmental complex matrices. The recoveries have been satisfactory, and in all of the samples the RSDs were lower than 20%. The method has been satisfactorily applied to real samples and three natural hormones (progesterone, testosterone and androstenedione) were detected in the range of $\mu g \cdot L^{-1}$. In wastewater samples were detected only two hormones over the quantification limits, progesterone in untreated hospital effluent and testosterone in secondary treatment samples. Finally, in osmosis treatment and tap water samples, no hormone under study was detected.

Acknowledgements

Rayco Guedes-Alonso thanks the University of Las Palmas de Gran Canaria (Spain) for his Ph.D. student grant. The authors would also thank to Emalsa S.A. for allowing the sampling in the wastewater system and the different wastewater treatment plants.

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Figure caption:

Figure 1. Response surfaces of 3^2 experimental design for the study of the extraction and desorption times.

Figure 2. Effect of sample pH in the extraction efficiency of the FPSE method.
* Values with the same letter are not statistically different at 5% significance level according to the Dunnett T3 nonparametric test.

Figure 3. Back-extraction efficiencies of Milli-Q and urine samples.

Figure 4. Analytical signal suppression/enhancement for wastewater and urine samples.

Figure 5. Chromatograms of (a) hospital untreated wastewater and (b) urine real samples.

Figure 1

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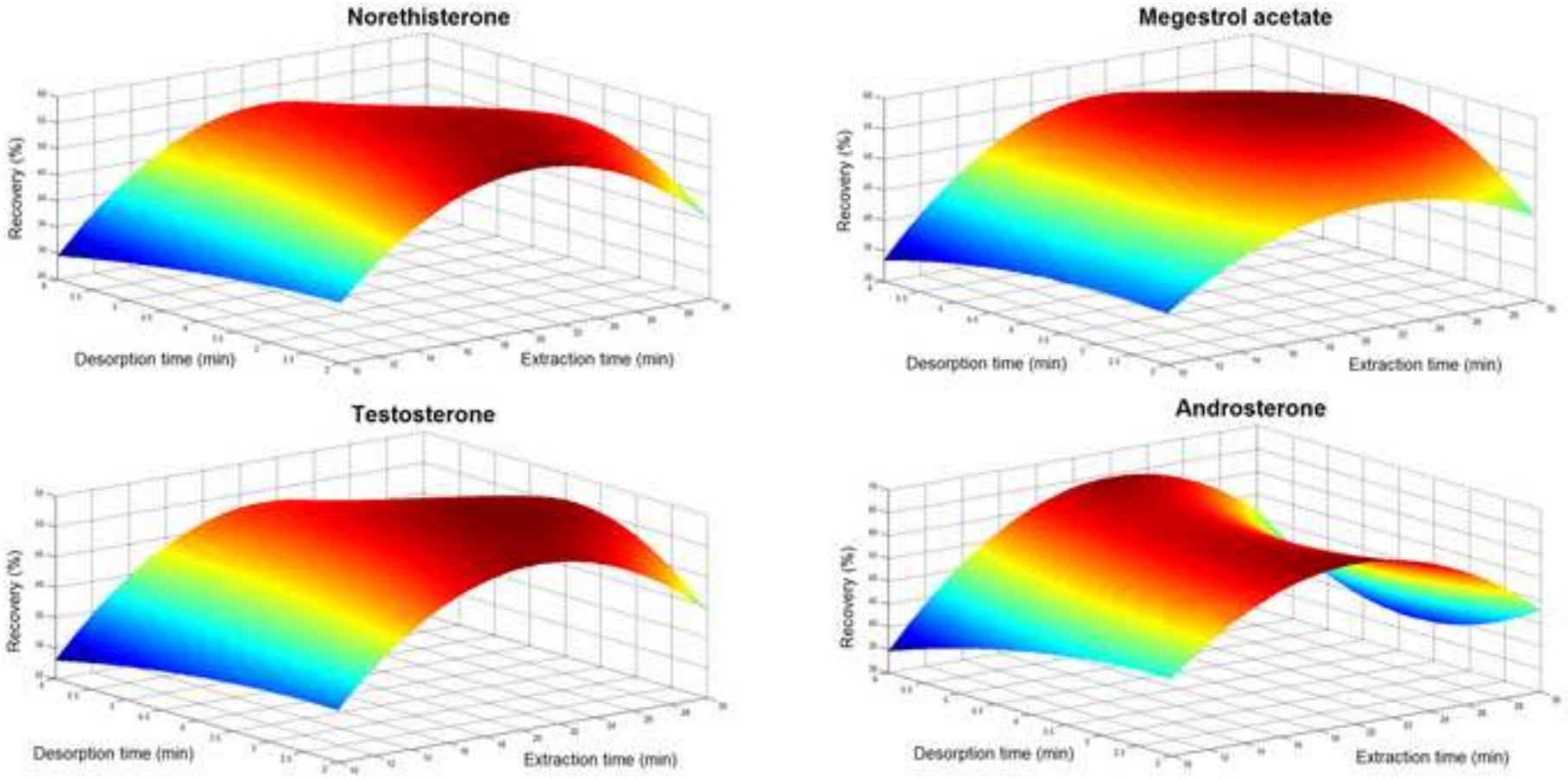

Figure 2

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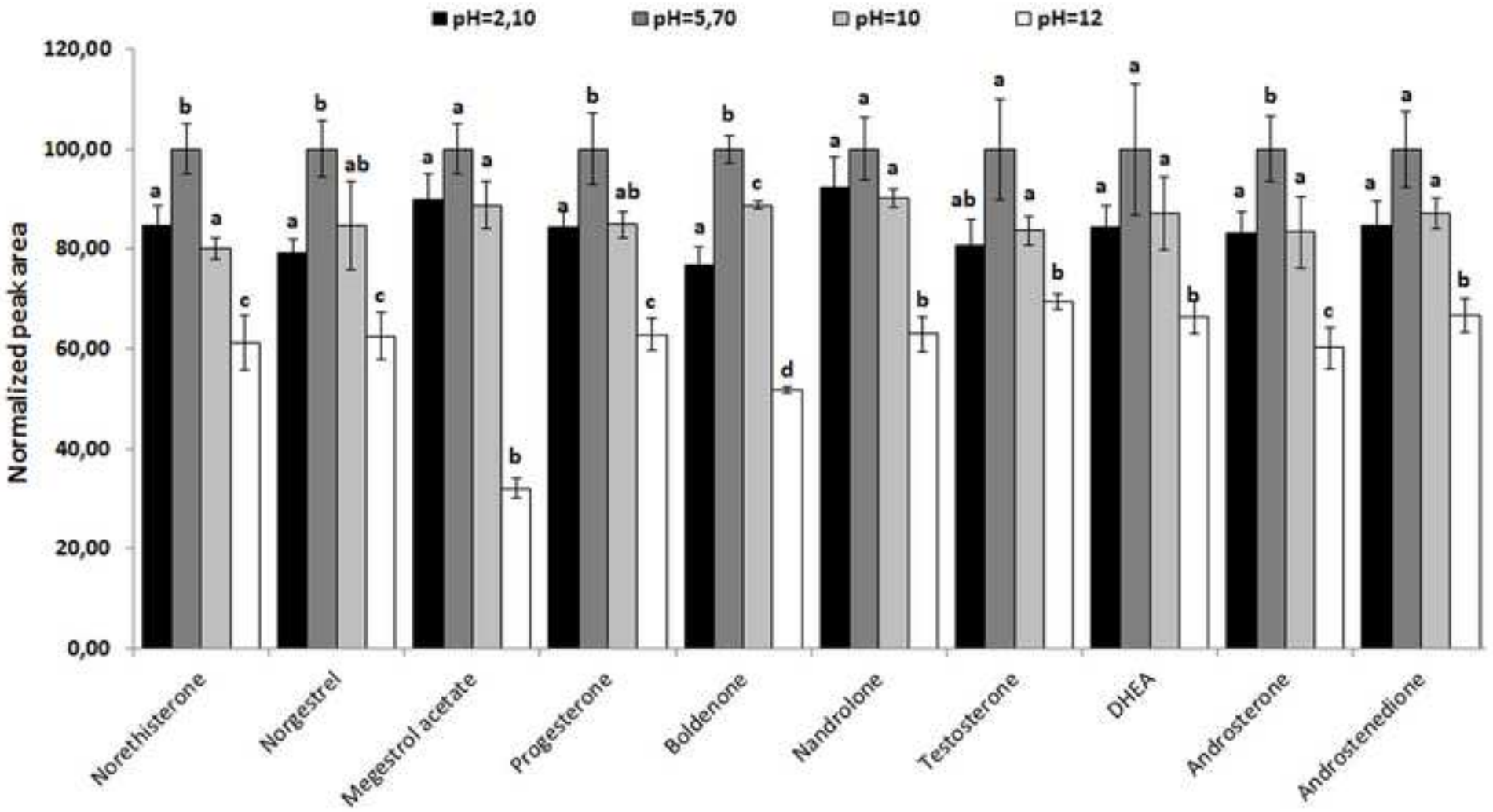

Figure 3

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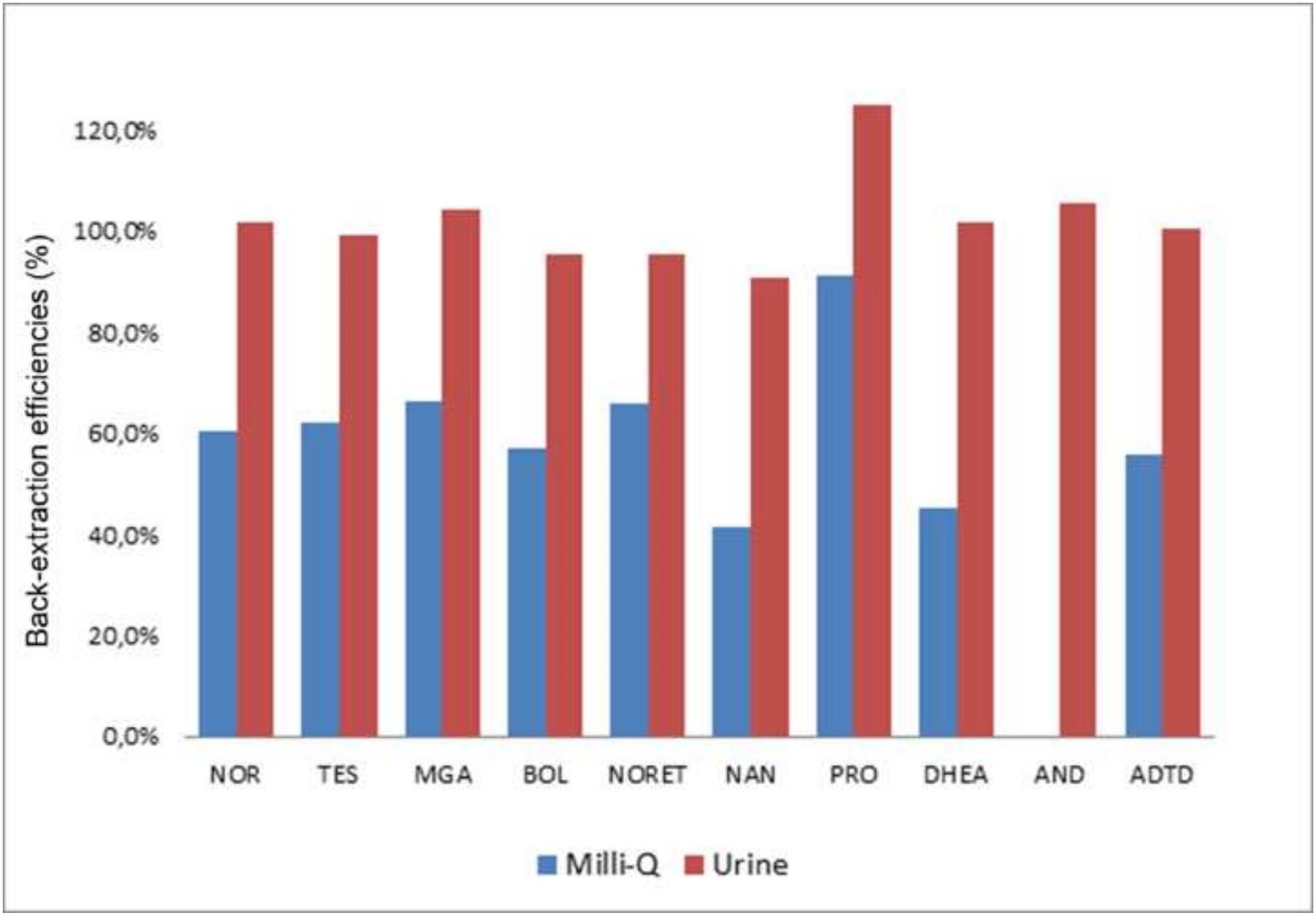

Figure 4

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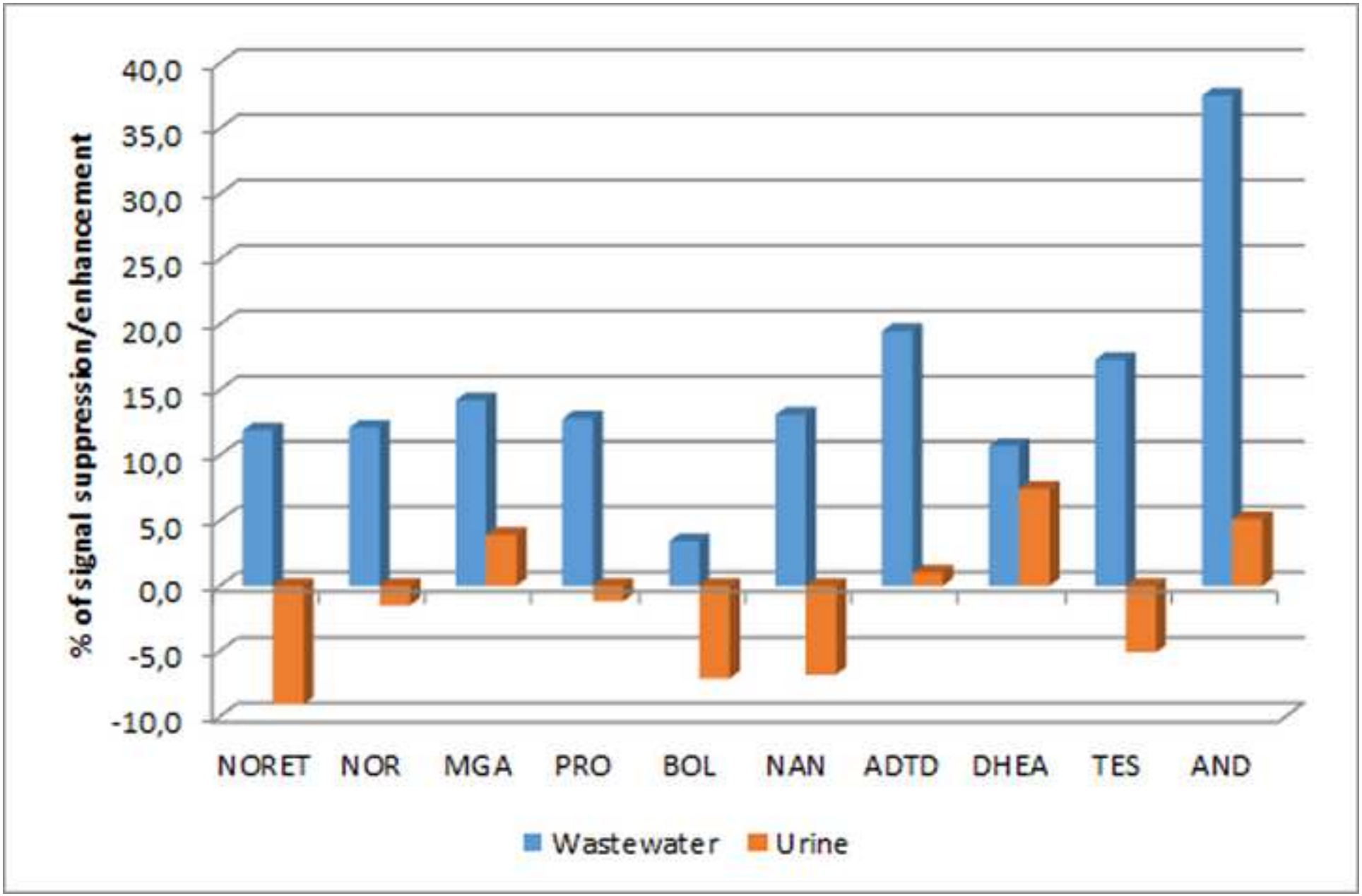

Figure 5

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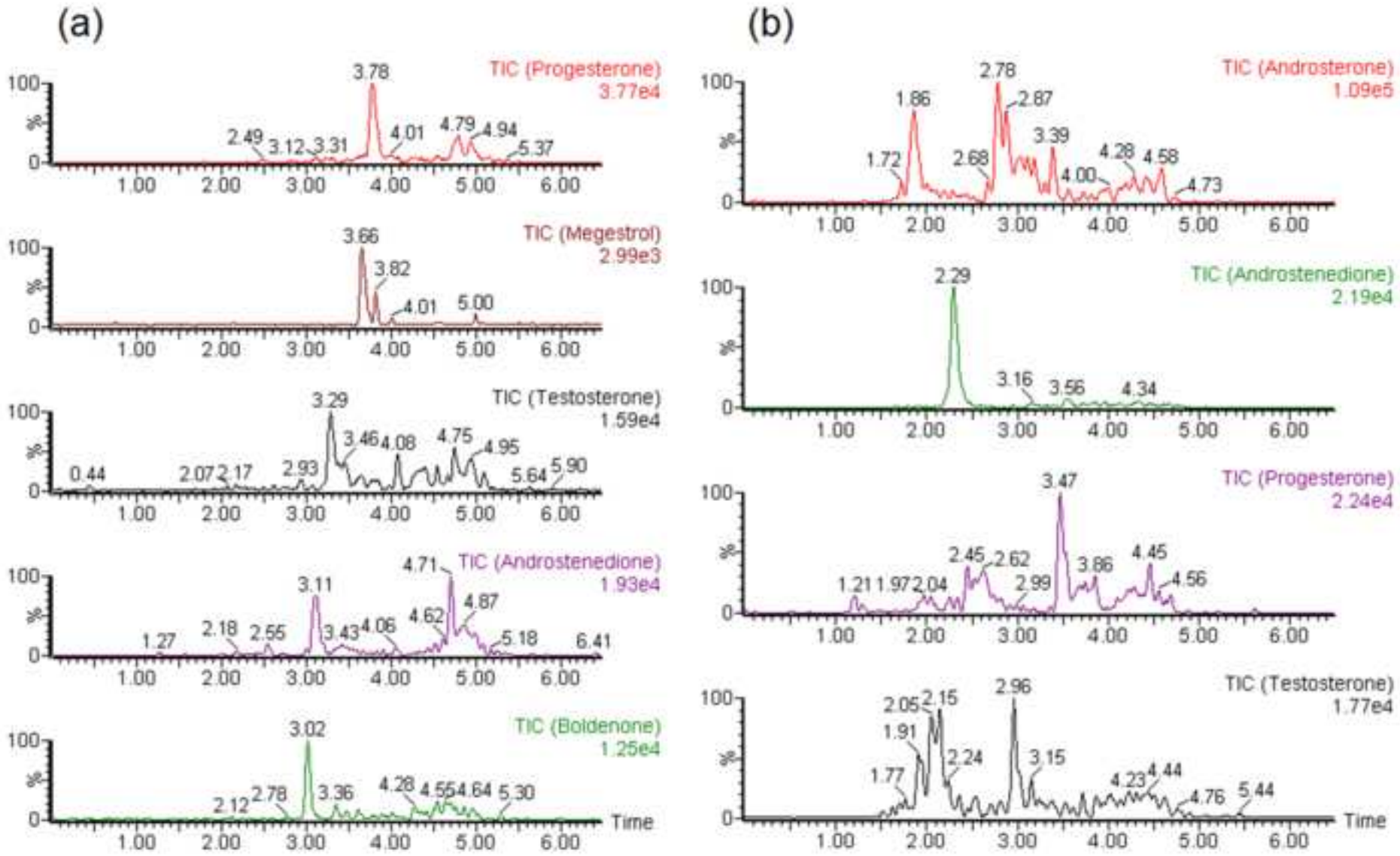
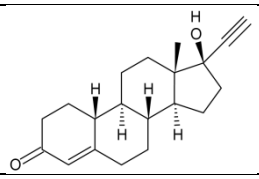
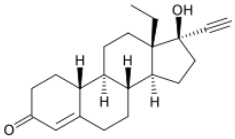
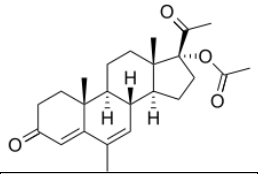
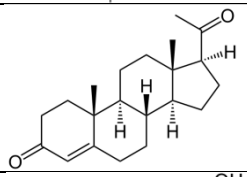
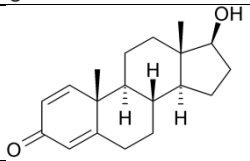
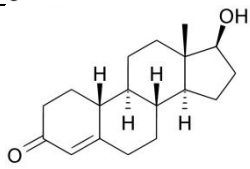
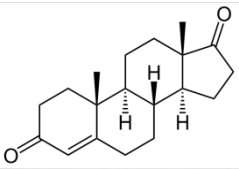
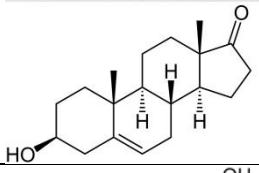
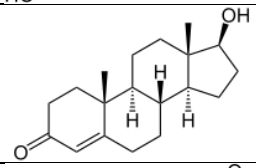
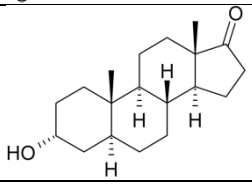


Table 1

Table 1. List of hormone compounds, structures, retention times and surrogate standards used.

Abbreviation	Compound	Structure	K_{OW}[a]	pKa[b]	t_R (min)	Internal standard
NORET	Norethisterone		2.97	13.09±0.40	2.80	Progesterone D9
NOR	Norgestrel		3.48	13.09±0.40	3.09	
MGA	Megestrol acetate		4.00	n.a[c]	3.35	
PRO	Progesterone		3.87	n.a[c]	3.47	
BOL	Boldenone		3.05	15.05±0.60	2.70	Testosterone D3
NAN	Nandrolone		2.62	15.06±0.40	2.78	
ADTD	Androstenedione		2.75	n.a[c]	2.79	
DHEA	Dehydroepiandrosterone		3.23	15.02±0.60	2.96	
TES	Testosterone		3.32	15.06±0.60	2.97	
AND	Androsterone		3.69	15.14±0.60	3.56	

[a] from EPI (Estimation Programs Interface) Suite™
[b] from Scifinder database
[c] not available

Table 2. Mass spectrometer parameters for the determination of target analytes

Compound	Precursor ion (m/z)	Cone voltage (Ion mode)	Quantification ion, m/z (collision potential, V)	Confirmation ion, m/z (collision potential, V)
NORET	299.2	30 V (ESI +)	109.1 (25)	91.0 (40)
NOR	313.2	38 V (ESI +)	109.0 (26)	245.1 (18)
MGA	385.5	30 V (ESI +)	267.3 (15)	224.2 (30)
PRO	315.3	30 V (ESI +)	97.0 (18)	109.1 (25)
BOL	287.2	30 V (ESI +)	121.0 (28)	135.1 (15)
NAN	275.2	35 V (ESI +)	109.1 (20)	83.0 (30)
ADTD	287.2	25 V (ESI +)	97.1 (20)	109.1 (20)
DHEA	289.2	20 V (ESI +)	91.0 (40)	157.1 (30)
TES	289.2	38 V (ESI +)	97.0 (22)	109.0 (21)
AND	291.2	20 V (ESI +)	199.1 (20)	91.0 (35)
Deuterated Compound	Precursor ion (m/z)	Cone voltage (Ion mode)	Quantification ion, m/z (collision potential, V)	Confirmation ion, m/z (collision potential, V)
PRO-d9	324.3	35 V (ESI +)	100.1 (20)	113.1 (20)
TES-d3	292.2	35 V (ESI +)	97.1 (25)	109.1 (20)

Table 3. Partial and bivariate correlations of the variables under study. Maximum and minimum values are +1 and –1.

Compound	Extraction time (min)	Ionic strength (% NaCl)	Desorption time (min)	Extraction time x Ionic strengt	Extraction time x desorption time
NORET	0.172	–0.463	–0.575	0.091	0.323
NOR	0.141	–0.519	–0.474	0.087	0.277
MGA	–0.158	–0.468	–0.712	–0.084	–0.162
PRO	–0.165	–0.663	–0.549	–0.148	–0.110
BOL	0.157	–0.460	–0.556	0.083	0.307
NAN	0.461	–0.307	–0.429	0.168	0.247
ADTD	–0.158	–0.833	–0.268	–0.240	–0.044
DHEA	0.447	–0.586	–0.269	0.362	0.440
TES	0.430	–0.626	–0.525	0.111	0.382
AND	–0.364	–0.636	–0.299	–0.322	–0.122

Table 4

Table 4. Analytical parameters of target analytes for the environmental water and urine samples

Compound	LOD[a] (ng·L^{-1})	Tap water						Osmosis effluent wastewater						Untreated effluent/biological treated wastewater					
		Relative Recovery (%) n=3		Intra-day RSD[b] (%) n=3		Inter-day RSD[b] (%) n=3x3		Relative Recovery (%) n=3		Intra-day RSD[b] (%) n=3		Inter-day RSD[b] (%) n=3x3		Relative Recovery (%) n=3		Intra-day RSD[b] (%) n=3		Inter-day RSD[b] (%) n=3x3	
		10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}	10 ng·mL^{-1}	50 ng·mL^{-1}
NORET	33.5	94.4	95.4	6.2	5.2	8.4	6.5	80.6	79.3	2.4	3.4	8.8	8.4	86.0	89.5	3.5	3.5	18.4	18.3
NOR	1.7	103.5	93.6	9.5	5.0	9.0	4.4	94.1	79.5	3.7	3.0	8.6	6.0	102.7	88.9	5.5	4.2	20.0	16.7
MGA	21.4	121.2	103.9	8.6	2.5	9.8	9.1	102.2	109.0	3.8	5.0	9.4	7.8	114.4	120.8	2.7	3.0	13.7	17.0
PRO	6.9	84.2	96.9	8.2	2.1	9.3	6.8	79.9	81.5	4.5	4.4	7.3	8.1	79.8	87.9	7.1	3.2	13.0	18.2
BOL	46.9	72.9	91.2	7.7	4.7	7.2	7.2	76.2	92.4	7.8	8.4	10.0	9.3	66.6	87.4	3.3	2.5	15.8	19.1
NAN	50.7	102.4	96.0	4.4	4.7	7.1	8.2	82.8	85.6	0.5	8.7	9.9	9.2	86.6	86.1	4.8	2.6	17.9	14.5
TES	2.2	81.4	91.8	6.4	4.4	6.7	5.4	76.6	75.6	0.8	3.2	7.9	9.2	78.7	83.5	1.1	3.0	13.0	18.0
DHEA	264	87.4	92.7	8.7	5.9	7.8	10.0	82.4	89.3	6.0	3.2	10.0	7.8	77.6	81.9	1.6	5.1	22.8	18.4
AND	63.6	98.9	83.8	11.5	4.6	5.4	9.9	92.2	70.0	7.2	1.2	7.6	9.8	98.1	83.9	1.1	2.7	18.6	17.1
ADTD	19.4	77.9	97,8	7.6	3.8	9.1	7.0	68.2	88.2	7.7	7.1	9.6	8.8	65.9	90.9	3.4	1.4	18.7	19.3

[a] Limit of detection

[b] Relative standard deviation

Table 4. (cont.) Analytical parameters of target analytes for the environmental water and urine samples

Compound	Urine				
	LOD[a] ($ng \cdot L^{-1}$)	Intra-day RSD[b] (%) n=3		Inter-day RSD[b] (%) n=3x3	
		10 $ng \cdot mL^{-1}$	50 $ng \cdot mL^{-1}$	10 $ng \cdot mL^{-1}$	50 $ng \cdot mL^{-1}$
NORET	35.2	1.8	3.1	18.0	8.3
NOR	132.3	4.3	4.2	18.1	11.5
MGA	11.1	6.5	9.9	13.0	10.7
PRO	12.8	7.1	6.4	16.9	9.9
BOL	37.9	6.2	4.5	18.8	10.2
NAN	50.1	9.0	7.2	15.6	8.4
TES	8.9	9.3	2.8	14.7	10.1
DHEA	110.6	4.9	1.4	17.2	9.7
AND	80.0	9.2	7.9	9.4	7.8
ADTD	25.6	5.2	4.7	20.0	10.4

[a] Limit of detection

[b] Relative standard deviation

Table 5. Comparison of different analytical methods for the extraction and determination of androgens and progestogens from environmental and biological samples.

Compounds	Matrix studied	Extraction technique	Determination technique	Detection limits	Reference
Norethisterone Norgestrel Progesterone	Surface waters Sea water Wastewater	Bar adsorptive microextraction (BAμE)	HPLC-DAD	80 – 100 $ng \cdot L^{-1}$	[33]
Norgestrel Testosterone	Surface waters Wastewater	Automated online solid-phase extraction	LC-MS/MS	2.5 – 10 $ng \cdot L^{-1}$	[15]
Androstenedione Androsterone Megestrol acetate Nandrolone Norgestrel Progesterone Testosterone	Surface water Wastewater	Solid phase extraction	LC-MS/MS	0.2 – 2.5 $ng \cdot L^{-1}$	[35]
Androstenedione Progesterone Testosterone	Wastewater	SPE	GC-MS	1 – 2 $ng \cdot L^{-1}$	[12]
Norethisterone Norgestrel Progesterone	Wastewater	On-line SPE	LC-MS/MS	20 – 50 $ng \cdot L^{-1}$	[17]
Boldenone Megestrol acetate Nandrolone Norethisterone Norgestrel Progesterone Testosterone	Wastewater	On-line SPE	UHPLC-MS/MS	0.5 – 4 $ng \cdot L^{-1}$	[34]
Nandrolone Progesterone Testosterone	Wastewater Seawater Surface water	SBSE	HPLC-DAD	110 – 180 $ng \cdot L^{-1}$	[18]
Androstenedione Androsterone Boldenone Nandrolone Norgestrel Progesterone Testosterone	Surface water Wastewater	SPE	LC-MS/MS	0.02 – 1.44 $ng \cdot L^{-1}$	[20]
Boldenone Nandrolone Testosterone	Urine	SPE	LC-MS/MS	170 – 290 $ng \cdot L^{-1}$	[26]
Androstenedione Androsterone Boldenone Dehydroepiandrosterone Megestrol acetate Nandrolone Norethisterone Norgestrel Progesterone Testosterone	Tap water	FPSE	UHPLC-MS/MS	2 – 60 $ng \cdot L^{-1}$ DHEA: 260 $ng \cdot L^{-1}$	This study

Table 6. Concentration of hormones found in real environmental and biological samples

Compound	WWTP Secondary effluent ($ng \cdot L^{-1}$)	Hospital untreated wastewater ($ng \cdot L^{-1}$)	Urine ($\mu g \cdot L^{-1}$)
Norethisterone	n.d.[a]	n.d.[a]	n.d.[a]
Norgestrel	n.d.[a]	n.d.[a]	< LOQ[b]
Megestrol acetate	< LOQ[b]	< LOQ[b]	n.d.
Progesterone	< LOQ[b]	227.3	1.1
Boldenone	< LOQ[b]	< LOQ[b]	n.d.[a]
Nandrolone	n.d.[a]	n.d.[a]	n.d.[a]
Testosterone	28.3	< LOQ[b]	2.3
DHEA	n.d.[a]	n.d.[a]	n.d.[a]
Androsterone	n.d.[a]	n.d.[a]	< LOQ[b]
Androstenedione	< LOQ[b]	< LOQ[b]	3.5

[a] Not detected

[b] Value below the quantification limit