APPLICATION OF MICROWAVE-ASSISTED EXTRACTION AND ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY FOR THE ANALYSIS OF SEX HORMONES AND CORTICOSTEROIDS IN SEWAGE SLUDGE SAMPLES

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Abstract

Hormonal compounds are a concern to the international community because they can affect to aquatic biota and are therefore considered to be endocrine disrupting compounds. These compounds have lipophilic properties, so they tend to accumulate in solid matrices, such as sewage sludge. This work presents the optimization of a microwave-assisted extraction process combined with ultra-high performance liquid chromatography-tandem mass spectrometry for the determination of fifteen hormonal compounds in sludge samples. The proposed method has relative standard deviations below 23%, good recoveries (over 71%) for all compounds, detection limits that ranged from 1.1 to 7.9 $ng \cdot g^{-1}$ and quantification limits which ranged from 3.7 to 26.3 $ng \cdot g^{-1}$. The method was used to analyse sludge samples from four different wastewater treatment plants of Gran Canaria (Spain) with different wastewater treatments. 17β-estradiol, 17α-ethynylestradiol, norgestrel and cortisone, were detected in sludge samples at concentrations that ranged from 17.3 to $1.44 \cdot 10^3$ $ng \cdot g^{-1}$. The developed method permits the use of small quantities of sample and organic solvents, presents short extractions times and is the first one based on microwave-assisted extraction for the analysis of both sex hormones and corticosteroids.

Keywords

Mass spectrometry, Microwave-assisted extraction, Sludge, Steroid hormones, Ultra-high performance liquid chromatography

1. Introduction

Steroid hormones are an important group of active molecules that are involved in most of the physiological functions of the human body, from cerebrovascular function to metabolism, accumulation and distribution of adipose tissues, salt and water balance and the development of sexual characteristics [1,2]. All steroid hormones are based on the same structure, and they are classified into five sub-families depending on the radicals of the molecule. Oestrogens have a structure of 18 carbon atoms, whereas androgens have 19 and progestogens have a basic structure based on 21 carbons.

Moreover, steroid hormones are prescribed in many health disorders because it is necessary to stabilize hormone homeostasis or because of the properties of the steroid hormones in the different vital functions. Different families of steroid hormones are used in hormone-replacement therapies, as contraceptives, to promote strength, mass and muscular size, as anti-inflammatories and to treat immunosuppressive effects on metabolism [3–5]. Steroid hormones are excreted as free or conjugated compounds, and they reach wastewater treatments plants (WWTPs). The removal of these compounds is mainly produced by biodegradation and adsorption on sludge [6–9] but, in most cases, they are not completely removed from the wastewater. In fact, the elimination efficiencies are usually in the range from 50 to 90%

The concentrations of steroid hormones in water samples (both wastewater and natural samples) and the effects of steroids on aquatic biota exposed to water streams, with measurable quantities of sex hormones as a WWTP effluent, have been studied [4,6,10–13]. WWTPs and unusual water streams, such as hospital effluents, are considered the main sources of environmental contamination by steroid hormones [14,15]. Nevertheless, in comparison with the studies about water samples, the studies about the presence of steroid hormones in environmental solid matrices are scarce, and most of the studies evaluate the presence of only one sub-family of steroid hormones in the sludge or sediment [16–18].

The main challenge for the analytical scientists is the development of analytical methods that permit the separation of steroid hormones from the samples (and even other interferences),

especially from the solid samples, which is difficult because natural and synthetic hormones are moderately lipophilic and highly sorptive [19]. The first methods developed in the 1980s and the early 1990s were based on Soxhlet extraction and steam-distillation [16], but these methods were too time-consuming and produced large amounts of organic solvent waste. To resolve these main drawbacks, different extraction methods were developed, and the most commonly used extraction technique, due to its simplicity, was ultrasonic-assisted extraction (UAE) [17,20]. UAE resolves many disadvantages of the classical extraction techniques, mainly using shorter extraction times, but the use of a large amount of hazardous solvents, such as acetone, methanol and dichloromethane, was not resolved. For this reason, some extraction techniques, such as accelerated solvent extraction (ASE) [18,21,22], or microwave assisted extraction (MAE) [16,19,23], have been developed for the extraction of steroid sex hormones from environmental solid matrices.

In the last 15 years, microwaves have been widely used to accelerate sample digestion and chemical reactions and to extract organic compounds (especially thermal-stable compounds) from environmental and edible matrices [24]. MAE have many advantages, such as the use of small quantities of sample and organic solvents, shorter extractions times and the capability of analysing a large quantity of samples in a single run. The use of MAE to extract different families of steroid hormones from solid matrices has been developed, especially to extract oestrogens, but there are few studies that have developed an analytical methodology focused on the extraction of the different families of steroid hormones from complex environmental matrices, such as wastewater sludge.

The aim of this work is to develop an analytical methodology based on microwave-assisted extraction coupled to ultra-high performance liquid chromatography with tandem mass spectrometry detection (MAE-UHPLC-MS/MS) for the analysis of 15 hormonal steroid compounds in sludge samples. To develop the method, all of the parameters that affect the extraction method, such as the extraction time, the microwave power and the extractant volume, have been optimized following a statistical experimental design. Once optimized, the method is

used for the analysis of sludge samples from four different wastewater treatment plants of Gran Canaria (Spain) that use different treatment processes and are different sizes.

2. Materials and methods

2.1 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 ammonia to adjust the pH of the mobile phase, were obtained from Panreac Química (Barcelona, Spain). All of the steroid hormones (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.

2.2 Sampling

Sludge samples were collected from the secondary treatment of different wastewater treatment plants (WWTPs) of different sizes on the island of Gran Canaria (Spain). WWTP1 and 2 treat the wastewater of two high-density areas of the island with equivalent populations of 260000 and 130000, respectively. The sludge of WWTP3 and 4 comes from two small WWTPs that treat the wastewater of two rural zones and are designed for a population equivalent of 5000 and 7000 respectively. WWTP 1, 2 and 3 treat the wastewater using a traditional activated sludge treatment (AST), whereas WWTP4 is based on a membrane bioreactor.

The samples used for the optimization of the method were collected from WWTP1 in June of 2015 in glass jars that were rinsed beforehand with methanol and ultrapure water. The samples used for the validation of the method were collected in December of 2015 and January of 2016. After collecting the sludge samples, they were freeze-dried at -50°C using a LyoQuest

laboratory freeze dryer from Telstar (Barcelona, Spain), and the samples were stored in the dark at 4°C and were extracted within 48 hours.

2.3 Instrumentation

The analysis was performed by using an ultra-high performance liquid chromatography system coupled to a triple quadrupole mass spectrometer (UHPLC-MS/MS). The system consists of an ACQUITY Quaternary Solvent Manager, an autosampler, a column manager and a triple quadrupole detector (TQD) with an electrospray interface (ESI), which were all from Waters (Barcelona, Spain). These components were controlled using the MassLynx Mass Spectrometry Software. The microwave oven used to perform the extractions was a Multiwave Microwave Sample Preparation System equipped with a 6 EVAP rotor and 6 MF100 vessels (Anton Paar, Graz, Austria).

2.4 Chromatographic and detection conditions

The chromatographic separation was performed using an ACQUITY UHPLC BEH Waters C_{18} analytical column (50 mm × 2.1 mm, 1.7 µm particle size) from Waters, (Barcelona, Spain) operated at a temperature of 30°C. The injected sample volume was 10 µL, and the analyte separation was performed using water with 0.1% (v/v) ammonia to promote the ionization of the compounds in the electrospray interface of the detector, 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 then to 25:75 (v/v) in 1.25 minutes. Then, the gradient changed to 0:100 (v/v) in 1 minute. Finally, the mixture returned to 80:20 in 2.25 minutes and remained at that composition for pressure equilibration for an additional 0.5 minutes. Thus, the chromatographic separation was completed in 6.5 minutes.

For the quantitative analysis, the multiple reaction monitoring (MRM) parameters were optimized for each compound. The precursor ions for the oestrogens were $[M-H]^-$ in negative ion mode (ESI–) and were $[M+H]^+$ in positive ion mode (ESI+) for androgens, progestogens

and glucocorticoids. To optimize the quantification and confirmation fragment ions and the detection parameters of each compound (Table 2), a standard of 1 $mg \cdot L^{-1}$ in methanol was directly infused in the mass spectrometer at a flow rate of 10 $\mu L \cdot min^{-1}$, keeping in mind the composition of the mobile phase because the optimization was carried out combining the mobile phase with modifiers and the direct infusion of the standard. The electrospray ionization parameters were as follows: the capillary voltage was 3.5 kV in positive mode and –2.5 kV in negative mode, the source temperature was 150°C, the desolvation temperature was 500°C, and the desolvation gas flow rate was 1000 $L \cdot hr^{-1}$. Nitrogen was used as the desolvation gas, and argon was used as the collision-induced dissociation gas at a flow rate of 0.15 $mL \cdot min^{-1}$. The extractor and RF lens voltages were 3 V and 0.5 V, respectively, in both ionization modes.

In Figure 1, total ion current (TIC) chromatograms of the target compounds and internal standards at a concentration of 200 $\mu g \cdot L^{-1}$ is shown.

2.5 Microwave-assisted extraction procedure

To perform the extraction of the target analytes, 100 mg of lyophilized sludge was placed in microwave polytetrafluoroethylene (PTFE) vessels, and 10 mL of methanol was added as extractant. Then, the vessels were properly closed and placed in the microwave rotor in a symmetric arrangement. Once the rotor was placed in the microwave, the extraction was conducted with the following program: in the first minute, the rotor spun without microwave power to prepare the samples for the extraction. After this minute, a power of 500 W for 4 minutes was used to perform the extraction of the analytes from the sludge. Under these conditions, the methanol used as an extractant reached 65°C. Finally, the vessels were completely cooled for 5 minutes using the microwave fan and were then left at room temperature for another 10 minutes. Next, the extracts were filtered using 0.20 µm syringe polyethylene terephthalate (PET) filters from Macherey-Nagel (Düren, Germany). The filtered extracts were evaporated under a nitrogen steam and were reconstituted with 1 mL of methanol.

3. Results and discussion

3.1 Optimization of the microwave-assisted extraction

MAE is strongly affected by several parameters, such as the composition and volume of the extractant, the power of the microwave and the time of the extraction. To study the optimum conditions, an experimental design was used for the most dependent variables, which are the volume of the methanol used as extractant and the power and time used to develop the extraction. A 2^3 factorial experimental design (3 variables: power, irradiation time and extractant volume, at 2 different levels) was used to study the significance of each variable and the correlation/interaction between them. Then, another 3^2 experimental design was built with the two variables having strong partial correlation at three different levels to find the optimum extraction conditions. To compare the results of the different runs, a triplicate of a 100 mg of spiked sample (at an initial concentration of 2.5 $\mu g \cdot g^{-1}$) was used.

3.1.1 *2^3 factorial design*

The first factorial design was used as an initial screen of the contribution of the three variables one-by-one as well as between them to determine the extraction efficiency. The runs were randomized to avoid possible carry-over effects, and the three parameters were evaluated at two levels: power (100 and 500 W), extraction time (2 and 10 minutes) and extractant volume (2 and 10 mL of organic solvent). Power and extraction time are intrinsically bound to the extraction capacity, whereas the quantity of extractant is, sometimes, proportional to the amount of compound extracted. Pareto charts were built for each compound, and as can be observed in Figure 2 for several compounds, the greatest effects on the extraction were contributed by the extraction time and power as isolated variables. The combination of these two variables had the greatest effect. Moreover, the bivariate correlation of the extractant volume had a positive correlation (over 0.4 for most of the compounds), so a volume of 10 mL of methanol was fixed as the optimum value. According to the bivariate correlations and the Pareto charts of the effects, a second experimental design was created for the irradiation power and extraction time.

3.1.2 3^2 factorial design

In this factorial design, the two selected variables were evaluated at three levels to establish the optimum conditions for the extraction. Each run was conducted with three samples, and the levels studied were: 100, 300 and 500 W for the irradiation power and 2, 6 and 10 minutes for the extraction times. As in the previous experimental design, the experiments were randomized, and the whole design consisted in 11 runs because the central point of the statistical grid was analysed three times. No higher power or times were considered because at 500 W for 10 minutes, the extractant solvent was almost evaporated and the accuracy of the method decreased significantly. Using the results, response surfaces were built using Minitab 17 for each compound. As seen in Figure 3, the peak areas decreased with increasing extraction time; the highest peak area values were achieved at short times, especially at 100 and 500 W. Because the surfaces were different for the different families of compounds, it was necessary to reach a compromise and to select the two values that presented good analytical signals for all target compounds. In this sense, the optimum values were a power of 500 W for 4 minutes of extraction using an extractant volume of 10 mL of methanol.

3.2 Evaporation losses and matrix effects

It is important to evaluate the steps of the analytical method that could produce a loss in the analytical signal. To concentrate the extracted compounds, an evaporation step was necessary and was performed using nitrogen steam provided by a nitrogen generator from Cinel (Padova, Italy). To evaluate the losses produced in the evaporation step, the extracts were spiked with the target analytes at three concentration levels (5, 25 and 250 $\mu g \cdot L^{-1}$) before and after the evaporation, and the ratio doped before/doped after was evaluated for each compound. As seen in Table 3, the losses were not significant and were in the range of 2 to 39% for the compounds under study. Only diethylstilbestrol and ethynylestradiol had significant higher concentrations (over 15% but no more than 30%) after the evaporation step. This phenomenon occurred

because these compounds have worse ionization, and the interferences of the chromatogram could affect the quantification of their peak areas.

Regarding the matrix effect, in the analysis of complex matrices, such as sediment or sludge extracts, it is common to suffer signal suppressions, especially when electrospray ionization is used in the mass spectrometer detector because of the interferences extracted along with the target compounds. To evaluate the signal suppression caused by the matrix effects, the algorithm of Vieno et al.[26] was used (Eq. (1))

$$Signal\ suppression = \frac{A_s - (A_{sp} - A_{usp})}{A_s} \times 100 \quad (1)$$

In the equation, A_s corresponds to the peak area of the analyte in the pure standard solution, A_{sp} corresponds to the peak area in the spiked matrix extract and A_{usp} corresponds to the matrix extract of a real sample. The matrix effect was evaluated at three concentration levels (5, 25 and 250 $\mu g \cdot L^{-1}$)

The signal suppression was moderate for most of the compounds because the different interferences hindered the ionization of the target hormones due to the complexity of the matrix studied. In this sense, the ion suppressions were higher at the high concentration level and ranged, for most compounds, between 50 and 80%. For the other concentration levels, the ionic suppressions were variable and ranged from –91 to +3% for most compounds, except estriol, oestrone and diethylstilbestrol, which had signal enhancement of 25-46% at the level of 25 $\mu g \cdot L^{-1}$. To overcome the different matrix effects of the samples, three internal standards were added to the extracts obtained in the microwave-assisted extraction at a concentration of 200 $\mu g \cdot L^{-1}$. For the analysis of oestrogens, a C_{18} carbon structure of deuterated oestrogen (oestrone D2) was used as the internal standard, whereas testosterone D3 (C_{19} structure) was used for the analysis of androgens and progesterone D9 (C_{21} structure) was used for the analysis of progestogens and glucocorticoids.

3.3 Analytical parameters

The linearity, recovery, repeatability, limits of detection and limits of quantification of the MAE-UHPLC-MS/MS method were evaluated to ensure the precision, accuracy and selectivity of the developed method. External calibration curves were prepared in the range between 0.5 and 400 $\mu g \cdot L^{-1}$ of each compound. Moreover, three internal standards (oestrone D2, 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 the areas and concentrations of compounds and internal standards, with correlation coefficients (r^2) higher than 0.995 both in external calibration curves and spiked sludge samples.

The recoveries of the method were studied by comparing six extracts of spiked sludge samples with six non-spiked sludge extracts spiked after the microwave extraction. This comparison was performed at three concentration levels (50, 250 and 2500 $ng \cdot g^{-1}$), and the results in Figure 4 show that the recoveries were good for all of the compounds (over 60% for all compounds). For ethynylestradiol, the recoveries were over 110%, which can be explained by the interferences extracted with target compounds.

The intra- and inter-day repeatability of the method was evaluated using six samples per day (Table 4). The samples were spiked with target compounds at the same three concentration levels. Both repeatability values were satisfactory, and the relative standard deviations were, in all cases, below 20%.

The method detection and quantification limits (MDL and MQL) for each compound were calculated as the concentration that generated a signal to noise ratio of 3 and 10 in the quantification ion transition. The detection limits were from 1.1 to 7.9 $ng \cdot g^{-1}$, and the quantification limits ranged from 3.7 to 26.3 $ng \cdot g^{-1}$. The calculated limits are appropriate for the analysis of hormonal compounds in sludge samples and are in the range of the limits reached by other studies. For example, as it is shown in Table 5, Vega-Morales et al. [16] developed a similar MAE method for estradiol-mimicking compounds and three oestrogens with similar limits of detection. However, this process required a clean-step based on solid-phase extraction and required a large quantity of sludge (3 grams per analysis). Herrero et al. [18] developed a

pressurized liquid extraction coupled to liquid chromatography and a clean–up step of SPE for the determination of 9 glucocorticoids in sludge samples. The limits of detection were slightly lower than the limits reached in this paper (0.5 – 1 $ng \cdot g^{-1}$), but the recoveries of the methods were very low (8 – 18%) in comparison with the recoveries achieved in this study. Liu et al. [13] developed a method based on ultrasonic-assisted extraction for the extraction of 28 steroids from the four sub-families of steroids, and the limits were slightly lower than the limits of this study. However, the quantity of sludge used was 5 times higher than the sludge used in this study. Finally, the developed method does not require complex sample pretreatments as derivatization [27], and this results in lower sample handling times and lower production of unwanted toxic residues. According to the bibliography, the developed MAE-UHPLC-MS/MS method is simpler than previous methods because it does not require a large amount of sample and organic solvent, it has the capability of analysing a large quantity of samples in a single run and clean-up or derivatization steps are not necessary for the determination of the different compounds, so the analysis are greener and are conducted in shorter times.

3.4 Analysis of hormonal compounds in sludge samples

The optimized method was used for the identification and determination of target hormones in different real samples of sludge from different WWTPs located in the North and East of the island of Gran Canaria (Spain). The studied WWTPs are very different between them, because WWTP1 and WWTP2 treat the water of big population areas (more than 100000 equivalent population) while WWTP3 and WWTP4 are located in rural zones with a lower population density. In fact, these WWTPs are designed and treat the water of a 5000-7000 equivalent population. According to the purifying techniques, WWTP1, WWTP2 and WWTP3 use a traditional activated sludge treatment while WWTP4 treats the wastewater using a membrane biorreactor system. As seen in Table 6 and Figure 5, only four compounds under study were detected in the different WWTP sludge samples: two oestrogens, 17α-ethynylestradiol and 17β-estradiol, one progestogen, norgestrel and one glucocorticoid, cortisone.

The sizes of the WWTPs and, subsequently, the volume of treated waters were not a correlation with the concentrations of hormones measured in the sludge samples. In fact, the concentrations detected in the two smallest WWTPs are similar to the concentration determined in the biggest ones. Concerning to the oestrogens, the highest detected concentrations were of 17α-ethynylestradiol, which ranged from 31.5 to $1.44 \cdot 10^3$ $ng \cdot g^{-1}$. The other detected oestrogen, 17β-estradiol cannot be quantified because, in all cases, the concentrations were below the quantification limit. The presence of these two hormones in most of the sludge samples could be explained by their K_{OW}, (4.15 for EE and 3.94 for E2) which are higher than the K_{OW} of other oestrogens [9] and they are according with the affinity adsorption scale determined by Ying et al. [28] which establishes that the adsorptions decreases in this way: EE > E2 > E1 > E3. However, Ren et al. [29] concluded that this adsorption change in activated sludge, 17α-ethynylestradiol has a high affinity of adsorption on sludge. Furthermore, the hydrophobic properties of membrane biorreactor sludge produce a better sorption [9] which could explain why the highest concentrations of 17α-ethynylestradiol are detected in the sludge samples of WWTP4.

The sorption studies of progestogens and glucocorticoids are scarce, but the high concentrations detected of norgestrel could be explained by its use as oral contraceptive in combination with 17α-ethynylestradiol. Moreover, the K_{OW} value (K_{OW} = 3.48) of norgestrel is similar to 17α-ethynylestradiol and 17β-estradiol [30,31], so, its behaviour and adsorption affinity could be similar. Norgestrel was detected in all the studied sludge samples at concentrations which ranged from 430 to $1.35 \cdot 10^3$ $ng \cdot g^{-1}$ and, as happened with 17α-ethynylestradiol, the highest concentrations were detected in WWTP4. Finally, cortisone was only detected in the sludge samples from the membrane biorreactor wastewater treatment plant (WWTP4) at a concentration of 17.3 $ng \cdot g^{-1}$.

4. Conclusions

A microwave-assisted extraction combined with ultra high performance liquid chromatography tandem mass spectrometry method for the determination of sex hormones and corticosteroids is

presented for the first time. In this study, the MAE-UHLPC-MS/MS method was optimized and successfully applied to sludge samples for the determination of a group of fifteen steroid hormones.

The developed method has good selectivity and sensitivity and presents low detection limits that range from 1.11 to 7.90 $ng \cdot g^{-1}$, which are appropriate for the analysis of endocrine disrupting compounds that are present in complex environmental solid matrices. The recoveries were satisfactory (over 71%), and the RSDs were lower than 23%.

According to the best of our knowledge, the developed method is the first one based in microwave-assisted extraction for the determination of steroid hormones from the four sub-families. Moreover, it does not require an additional clean-up step, permits the use of small quantities of sample and organic solvents, has shorter extractions times and has the capability of analysing a large quantity of samples in a single run. The method has been satisfactorily applied to real samples and four different steroid hormones (two oestrogens, one progestogen and one glucocorticoid) were detected at concentrations which ranged from 17.3 to $1.44 \cdot 10^{3}$ $ng \cdot g^{-1}$. The highest concentrations detected belonged to 17α-ethynylestradiol and norgestrel which are combined and usually used as oral contraceptives. The concentrations of the studied compounds in WWTP4, which uses a membrane biorreactor technology, were always higher than the concentrations detected in the activated sludge wastewater treatment plants. According the obtained results it seems that the hydrophobic properties of membrane biorreactor sludge could produce a better sorption of this hormones and thus, higher concentrations of hormones in the sludge.

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Conflict of interest

The authors declare that they have no competing interests.

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Figure captions:

Figure 1. Chromatograms of the compounds under study at a concentration of 200 $\mu g \cdot L^{-1}$.

Figure 2. Pareto charts of the standardized effects for a compound of each hormone sub-family under study.

Figure 3. Response surfaces of the 3^2 factorial design for testosterone, prednisolone, norethisterone, cortisone, boldenone and oestrone.

Figure 4. Extraction recoveries of the optimized method in sludge samples spiked at three concentration levels.

Figure 5. Chromatograms of the quantified compounds detected in the WWTP4 sludge sample.

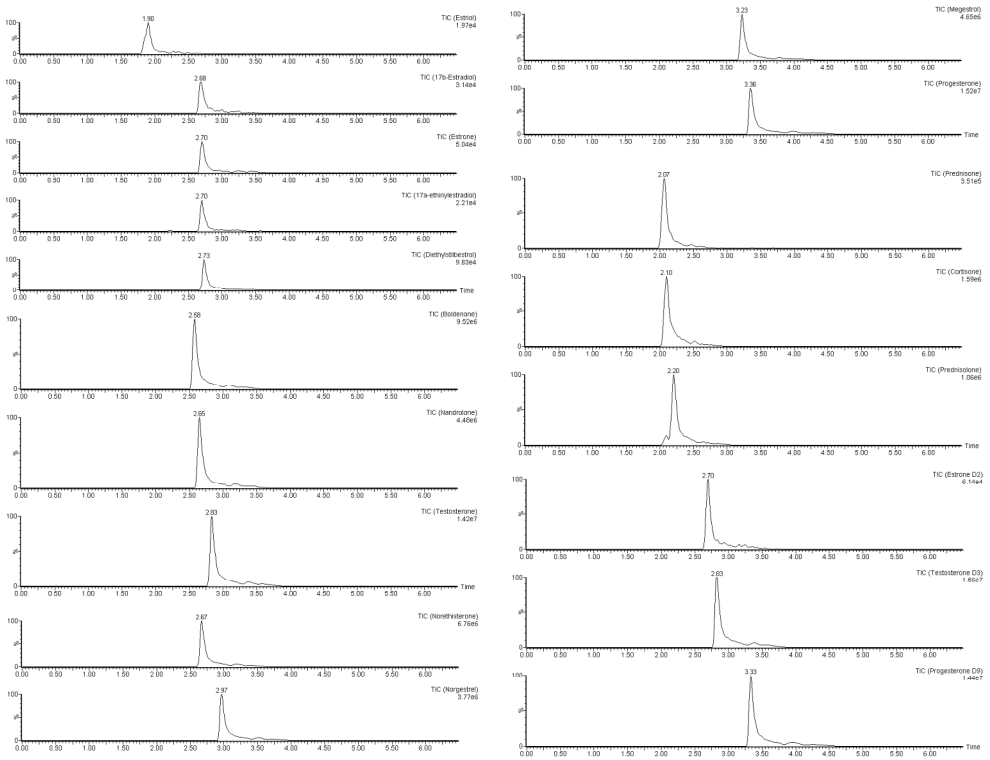

Figure 1. Chromatograms of the compounds under study at a concentration of 200 µg•L-1.

565x438mm (96 x 96 DPI)

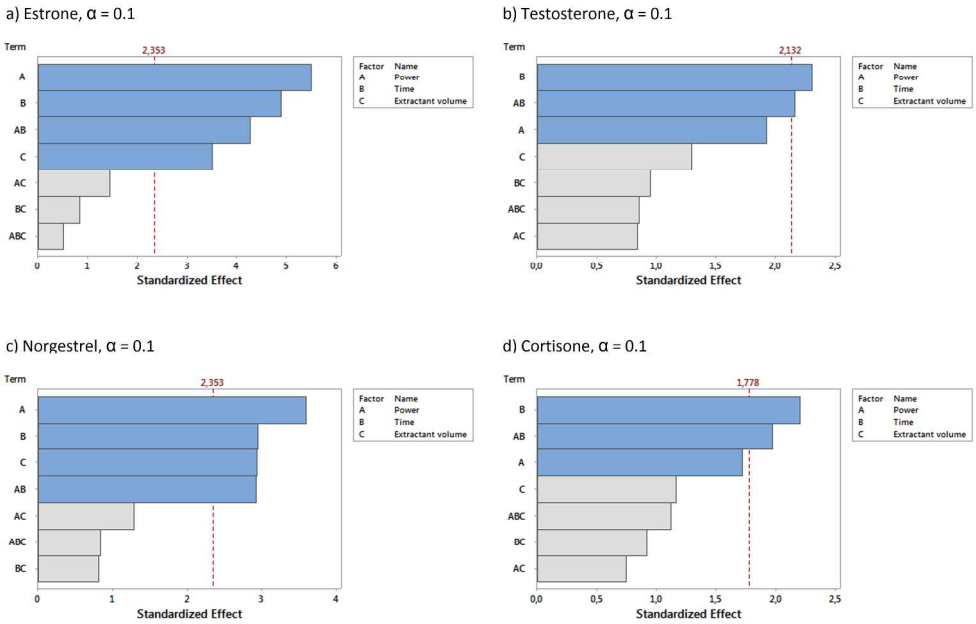

Figure 2. Pareto charts of the standardized effects for a compound of each hormone sub-family under study.

308x199mm (300 x 300 DPI)

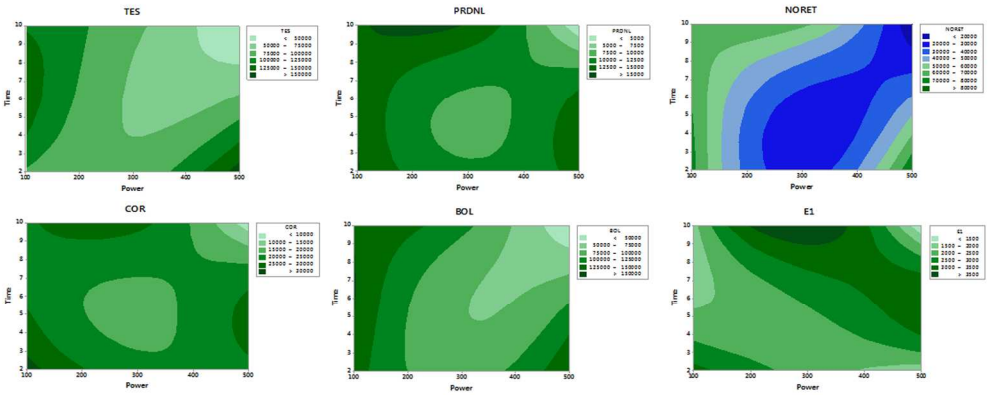

Figure 3. Response surfaces of the 32 factorial design for testosterone, prednisolone, norethisterone, cortisone, boldenone and oestrone.

238x98mm (300 x 300 DPI)

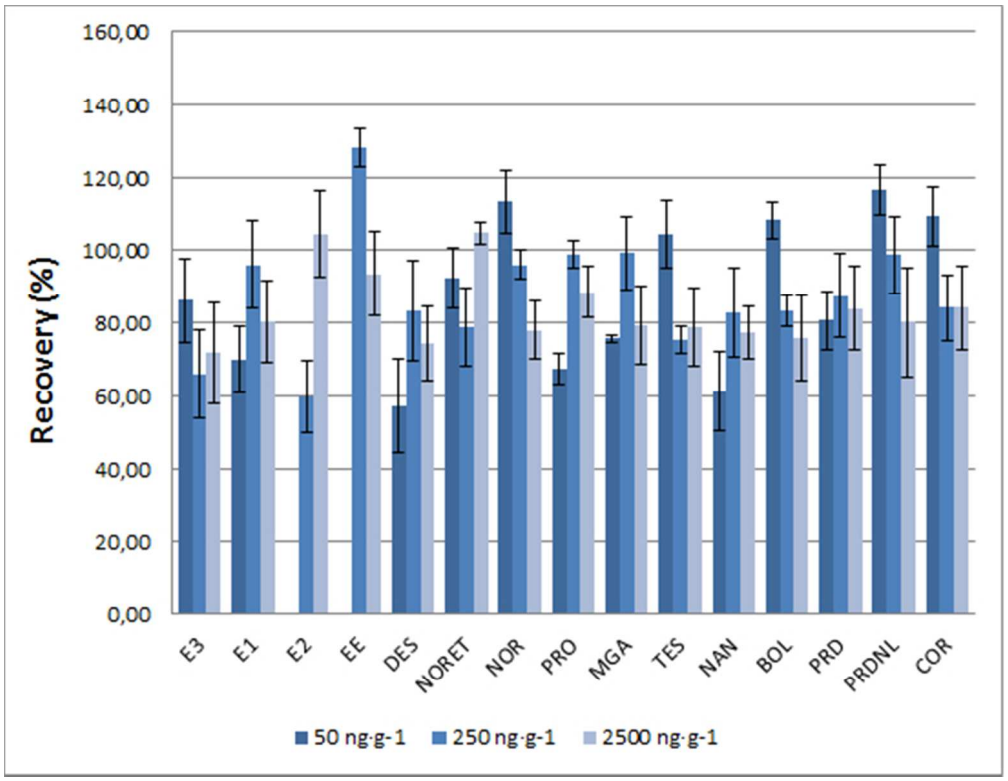

Figure 4. Extraction recoveries of the optimized method in sludge samples spiked at three concentration levels.

145x111mm (96 x 96 DPI)

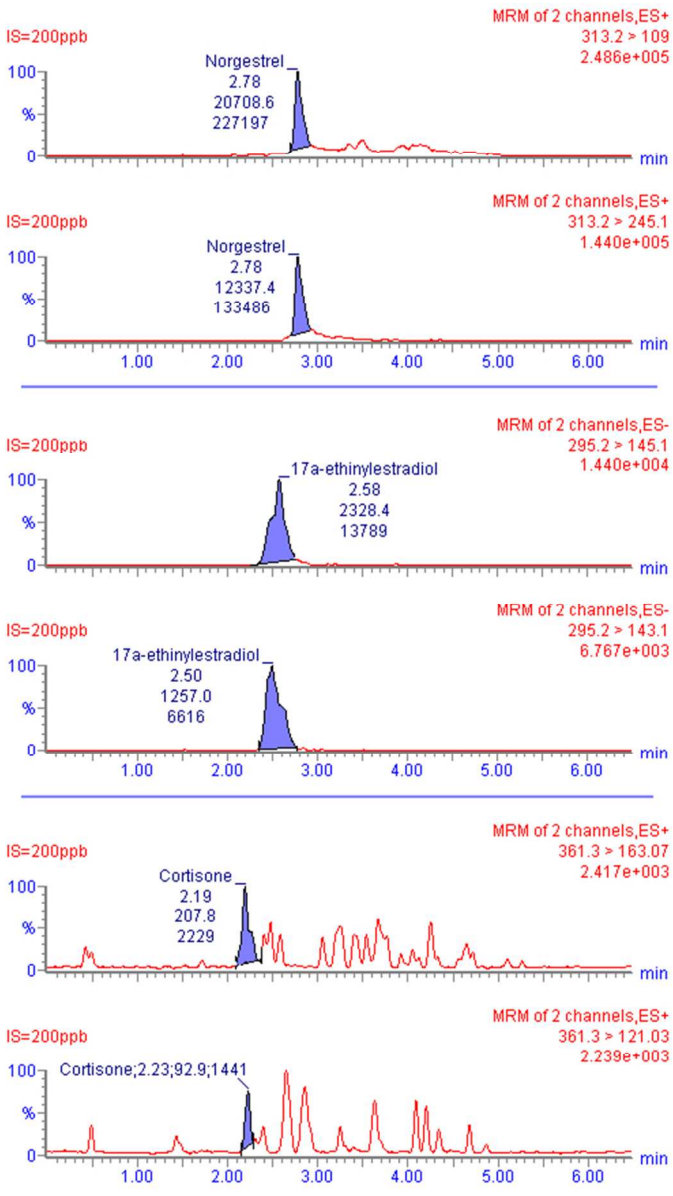

Figure 5. Chromatograms of the quantified compounds detected in the WWTP4 sludge sample.

133x233mm (96 x 96 DPI)

Table 1. Compounds under study and their abbreviations, internal standards used and retention times.

Type of hormone	Abbreviation	Compound	Internal standard	t_R (min)
Oestrogens	E3	Estriol	Estrone D2	1.91
	E2	17β-estradiol		2.69
	EE	17α-Ethynylestradiol		2.70
	E1	Estrone		2.71
	DES	Diethylstilbestrol		2.74
Progestogens	NORET	Norethisterone	Progesterone D9	2.67
	NOR	Norgestrel		2.97
	MGA	Megestrol acetate		3.23
	PRO	Progesterone		3.36
Androgens	BOL	Boldenone	Testosterone D3	2.58
	NAN	Nandrolone		2.66
	TES	Testosterone		2.84
Corticosteroids	PRD	Prednisone	Progesterone D9	2.07
	COR	Cortisone		2.10
	PRDNL	Prednisolone		2.21

Table 2. Quantification and confirmation fragment ions and their respective detection parameters for compounds under study.

Compound	Precursor ion (m/z)	Cone voltage (Ion mode)	Quantification ion, m/z (collision energy, V)	Confirmation ion, m/z (collision energy, V)	Quantification ion - confirmation ion ratio
E3	287.2	-65 V (ESI –)	171.0 (37)	145.2 (39)	0.98
E2	271.2	-65 V (ESI –)	183.1 (40)	145.1 (45)	0.79
EE	295.2	-65 V (ESI –)	145.1 (38)	143.1 (45)	0.34
E1	269.2	-65 V (ESI –)	145.0 (36)	143.0 (48)	0.21
DES	267.1	-50 V (ESI –)	237.1 (29)	251.1 (25)	0.88
NORET	299.2	30 V (ESI +)	109.1 (25)	91.0 (40)	0.61
NOR	313.2	38 V (ESI +)	109.0 (26)	245.1 (18)	0.68
MGA	385.5	30 V (ESI +)	267.3 (15)	224.2 (30)	0.80
PRO	315.3	30 V (ESI +)	97.0 (18)	109.1 (25)	0.78
BOL	287.2	30 V (ESI +)	121.0 (28)	135.1 (15)	0.65
NAN	275.2	35 V (ESI +)	109.1 (20)	83.0 (30)	0.67
TES	289.2	38 V (ESI +)	97.0 (22)	104.0 (21)	0.71
PRD	359.3	30 V (ESI +)	147.0 (15)	237.0 (20)	0.70
COR	361.3	30 V (ESI +)	163.0 (25)	121.0 (45)	0.18
PRDNL	361.3	20 V (ESI +)	147.1 (20)	173.1 (25)	0.33

Table 3. Evaporation losses and ion suppression/enhancement of the MAE-UHPLC-MS/MS method for a sludge sample spiked at three concentrations.

Compound	50 ng·g^{-1}		250 ng·g^{-1}		2500 ng·g^{-1}	
	Evaporation losses	Ion suppression	Evaporation losses	Ion suppression	Evaporation losses	Ion suppression
E3	n.a.[a]	-52.2	-2.12	46.7[c]	-27.3	12.8[c]
E2	n.a.[a]	n.a.[a]	-7.56	37.9[c]	-29.0	-85.1
EE	n.a.[a]	n.a.[a]	29.9[b]	3.77[c]	-36.4	-83.1
E1	-31.8	-61.4	9.44[b]	-14.1	-28.0	-79.9
DES	-21.7	-65.2	21.0[b]	26.2[c]	-38.9	-76.3
NORET	-19.6	-68.4	-8.55	-44.2	-3.11	122[c]
NOR	-13.3	n.a.[a]	-1.97	n.a.[a]	-23.2	-65.0
MGA	-37.7	-91.6	-3.23	-83.8	-28.9	-57.8
PRO	6.52[b]	-90.8	-21.8	-81.0	-31.4	-59.2
BOL	7.96[b]	-65.4	-17.6	-25.5	-31.3	-50.9
NAN	-7.45	-70.0	-5.36	-53.3	-28.5	-82.2
TES	-13.6	-81.6	-35.1	-81.0	-30.0	-74.4
PRD	26.3[b]	-22.9	3.94[b]	-1.02	-36.0	-45.2
COR	6.81[b]	-39.8	-9.99	3.45[c]	-34.4	-52.2
PRDNL	-31.48	-46.0	-23.4	2.93[c]	-30.4	-55.8

[a] not available

[b,c] a positive number indicates an enhancement of the analytical signal

Table 4. Analytical parameters of the optimised MAE-UHPLC-MS/MS method.

Compound	LOD[a] ($ng \cdot L^{-1}$)	MDL[b] ($ng \cdot g^{-1}$)	MQL[c] ($ng \cdot g^{-1}$)	Intra-day RSD[d] (%) n = 6			Inter-day RSD[d] (%) n = 3 x 6		
				50 $ng \cdot g^{-1}$	250 $ng \cdot g^{-1}$	2500 $ng \cdot g^{-1}$	50 $ng \cdot g^{-1}$	250 $ng \cdot g^{-1}$	2500 $ng \cdot g^{-1}$
E3	159.8	2.30	7.67	5.2	12.5	9.2	10.3	17.1	16.1
E2	58.2	5.57	18.57	n.a.[e]	18.3	13.5	n.a.[e]	15.4	15.2
EE	40.1	1.11	3.69	9.4	14.6	16.2	14.4	18.9	16.8
E1	139.5	1.48	4.92	11.3	16.9	9.9	21.3	19.3	19.5
DES	28.5	2.64	8.80	15.2	4.7	6.5	21.1	20.1	11.4
NORET	14.3	1.79	5.96	7.7	16.7	2.8	13.7	20.4	n.a.[e]
NOR	15.5	2.75	9.18	2.8	4.2	17.5	10.6	n.a.[e]	n.a.[e]
MGA	2.1	2.28	7.59	4.4	14.7	10.6	14.7	17.7	19.9
PRO	5.9	4.86	16.21	8.6	7.4	8.0	11.6	17.6	14.0
BOL	7.1	2.23	7.45	8.2	5.0	9.3	10.2	18.2	14.2
NAN	18.1	3.68	12.25	5.5	15.2	5.7	12.2	19.9	16.2
TES	9.7	1.59	5.30	6.7	8.4	9.2	15.4	18.4	17.8
PRD	24.4	7.90	26.33	9.3	12.3	5.9	16.3	20.2	13.0
COR	138.1	2.82	9.41	7.9	8.9	1.2	14.2	19.6	13.4
PRDNL	192.8	4.17	13.90	6.1	11.5	4.0	15.5	18.1	17.4

[a] Instrumental detection limit

[b] Method detection limit

[c] Method quantification limit

[d] Relative standard deviation

[e] Not available

Table 5. Comparison of different analytical methods for the extraction and determination of steroid hormones from sewage sludge samples.

Studied compounds	Sewage sludge weight	Extraction method	Clean-up step	Method detection limits ($ng \cdot g^{-1}$)	Method recoveries	Reference
E2, E3, EE	1000 mg.	MAE	SPE (Sep-Pak C18)	0.9-1.5	72-102%	Vega Morales et al. 2011
PRD, PRDNL, COR	1000 mg.	PLE	SPE (Bond Elut Plexa)	0.5	6-14%	Herrero et al. 2013
E1, E2, EE, DES, NOR, PRO, TES, NAN, BOL, PRD, PRDNL, COR	500 mg.	UAE	Silica gel cartridge	0.5-1.5	58-135%	Liu et al. 2011
E1, E2, E3, EE	100 mg.	UAE	SPE (Oasis HLB)	0.1-0.3	65-124%	Yu et al. 2011
E1, E2, E3, EE, DES, NOR, NORET, MGA, PRO, TES, NAN, BOL, PRD, PRDNL, COR	100 mg.	MAE	Not necessary	1.1-7.9	61-120%	This study

Table 6. Concentrations ($ng \cdot g^{-1}$) detected in sludge samples (n = 6) from the four WWTPs under study.

	EE	E2	NOR	COR
WWTP1	31.5 ± 7.10	< LOQ[a]	874 ± 66.7	n.d.[b]
WWTP2	315 ± 15.7	< LOQ[a]	430 ± 52.8	n.d.[b]
WWTP3	n.d.[b]	< LOQ[a]	981 ± 65.3	n.d.[b]
WWTP4	$1.44 \cdot 10^3$ ± 114	< LOQ[a]	$1.35 \cdot 10^3$ ± 74.4	17.3 ± 4.27

[a] Below quantification limit

[b] Not detected