Programa de Doctorado en Gestión Costera

Memoria para la Acreditación de la Etapa de Investigación (AEI)

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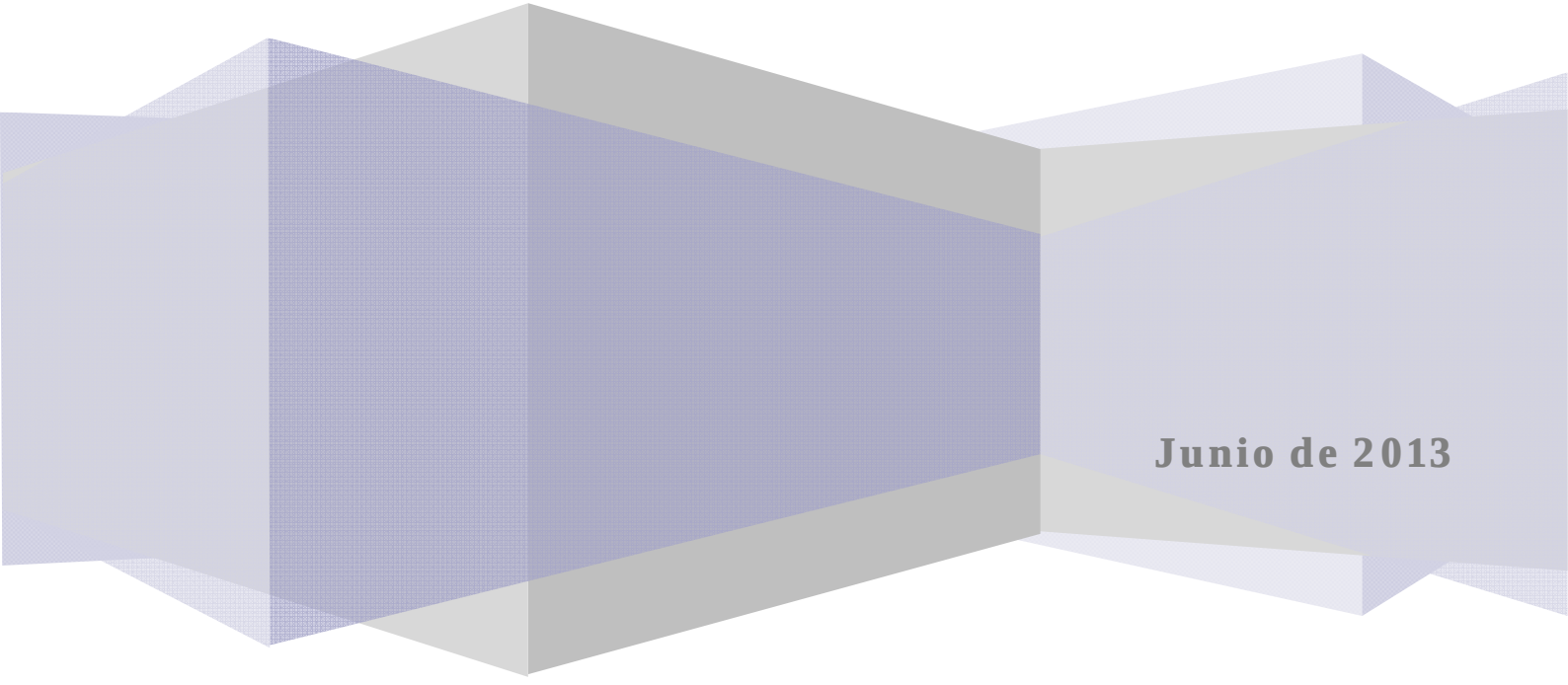

Junio de 2013

La presente memoria recoge la actividad investigadora realizada por el doctorando Rayco Guedes Alonso durante los últimos años, para obtener la Acreditación de la Etapa de Investigación, dentro del Programa de Doctorado en Gestión Costera de la Universidad de Las Palmas de Gran Canaria.

Dicha actividad investigadora se resume en forma de artículo científico, en el que se recogen las investigaciones realizadas desde septiembre de 2012, fecha de matriculación en el presente Programa de Doctorado. Además, se muestran también actividades científicas realizadas anteriormente pero ligadas a la investigación principal, conducente a la obtención del título de Doctor.

Dicha investigación principal se centra en la “Optimización y desarrollo de metodologías analíticas para la determinación de residuos hormonales en muestras de aguas, lodos, sedimentos y organismos de la isla de Gran Canaria”.

Esta investigación comenzó con la realización del Máster en Ciencia y Tecnología Química, mención en Química Analítica, impartido por la UNED durante el curso 2011-2012. Para la obtención de dicho título, se presentó la Tesina de Máster denominada: “Determinación mediante SPE – LC - MS/MS de compuestos hormonales en aguas depuradas procedentes de estaciones depuradoras de aguas residuales (EDARs)”. Además, en los últimos dos años se han presentado tanto artículos científicos como comunicaciones a congresos sobre los avances en dicha investigación.

A continuación se muestra el artículo científico que recoge las actividades investigadoras realizadas durante el curso académico 2012-2013:

Artículo científico

Determinación de quince compuestos hormonales en aguas depuradas usando extracción en fase sólida combinada con cromatografía líquida de "ultra" resolución con detección de espectrometría de masas (SPE-UHPLC-MS/MS)

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Resumen

Los compuestos hormonales son considerados contaminantes emergentes debido a que diversos estudios han determinado su presencia en muestras de aguas procedentes de estaciones depuradoras de aguas residuales (EDARs). En este estudio se ha desarrollado un método para la determinación simultánea de quince hormonas (cinco estrógenos, tres andrógenos, cuatro progestágenos y tres hormonas adrenocorticales) en muestras de influentes y efluentes de estaciones depuradoras. Debido a las bajas concentraciones de estos compuestos en el medioambiente, se ha elegido la extracción en fase sólida como procedimiento de extracción y preconcentración de los analitos. La determinación y cuantificación se ha realizado mediante cromatografía líquida de "ultra" resolución, acoplada a un detector de espectrometría de masas (UHPLC-MS/MS). El método desarrollado presenta unos límites de detección satisfactorios (entre 0.04 y 1.63 $ng \cdot L^{-1}$), buenas recuperaciones (por encima del 80% para todos los compuestos) y bajas desviaciones estándar relativas (inferiores al 13%). Utilizando el método propuesto, se analizaron muestras procedentes de influentes y efluentes de dos estaciones depuradoras de aguas residuales de Gran Canaria (España), las cuales presentaron concentraciones de algunas hormonas en el rango de 5 a 190 $ng \cdot L^{-1}$.

Research Article

Simultaneous determination of fifteen hormonal compounds in treated waters using solid phase extraction with ultra-high performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS)

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Abstract

Hormonal compounds are considerated as emerging pollutants because of several studies have confirmed their presence in water samples of wastewater treatment plants (WWTPs). In this study, a quantitative method for the simultaneous determination of fifteen hormones (five oestrogens, three androgens, four progestogens and three adrenocortical hormones) has been developed to determine these compounds in influent and effluent wastewater samples. Due to the very low concentrations of target compounds in the environment, a solid phase extraction procedure has been used to extract and preconcentrate the analytes. Determination and quantification were performed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The method developed presents satisfactory limits of detection (between 0.04 and 1.63 $ng \cdot L^{-1}$), good recoveries (over 80% for all compounds) and low relative standard deviations (under 13%). Samples from influents and effluents of two wastewater treatment plants of Gran Canaria (Spain) were analyzed using the proposed method, finding several hormones in influent samples with concentrations ranged from 5 to 190 $ng \cdot L^{-1}$.

1. Introduction

Due to the human development, it is supposed that more than 100,000 different chemical compounds can be introduced in the Environment every day, many of them in very small quantities. Many of these compounds, named emerging pollutants, are not regulated as pollutants; they probably will be in the future because of their potential negative effect in the ecosystem. For 20 years, many articles have reported the presence of these "new compounds" in wastewater. [1-2].

The emerging pollutant origin is mainly anthropogenic, considering that the majority of these compounds are biologically active substances that are synthesized to use them in agriculture, industry and medicine. The main source of these emerging pollutants are the residual urban waters and the wastewater treatment plants effluents because many of these WWTPs are not designed or optimized to treat this kind of compounds [3]. The main characteristic of these pollutants is that it is not necessary to remain in the environment to cause negative effects, in view of the fact that their constant introduction in it offsets their removal or degradation [4].

One important group of these contaminants is the hormonal residues, which are considered Endocrine Disrupting Compounds (EDCs). These compounds are defined as chemical substances capable of altering the natural hormonal equilibrium producing harmful effects in animals, humans and their progeny [5]. Hormones can be differentiated in sexual hormones (oestrogens, androgens and progestogens) which are synthetized in the reproductive organs, and adrenocortical hormones. Some of them have limits in their use, but not a specific legislation [6].

The steroid hormones help controlling the metabolism, inflammations, immunological functions, water and salt balance, sexual development and the capacity of withstand illnesses [7].The main medical application is to birth control. The synthetic oestrogen most used to this is 17α-ethinylestradiol (EE) [8]. Progestogens are also used as hormonal contraceptive that can be combined with oestrogens. Androgens are frequently used by some sportsmen to increase their strength, mass and muscular size. Finally, adrenocortical hormones are used in treatments of collagen diseases, inflammatory and allergic upsets and other illnesses. The term steroid can be used for natural hormones produced by the body as well as for artificially produced medicines that increase the natural steroid effect.

In the last 50 years, the natural and synthetic hormone worldwide consumption has grown, as much as in human medicine as in cattle farming, and they become the most prescribed medicines [9].

A significant quantity of consumed oestrogens leaves the organism through excretions. For example, 17β-estradiol (E2) is oxidized rapidly, becoming an estrone (E1) that can turn into estriol afterwards (E3). Besides, the 17α-ethinylestradiol (EE) is excreted as conjugated [10].

As regards to emission sources, in the first place are the wastewater treatment plants (WWTPs) [11], and secondarily, cattle waste such as those leachates from dung and uncontrolled dumping [12]. Several studies made in the WWTPs have

reported that the treatment plants are capable of eliminate around 60% of hormones [13-15].

Measurement of hormone residues is a very difficult task not only due to the difficulty in measuring very low concentration, but also due to a very complexity of the samples. Therefore, use of mass spectrometer (MS) as detector coupled with chromatography techniques has become a powerful method for the analysis of these types of compounds at trace levels [5,16-18]. Consequently, LC-MS/MS is the principally chosen technique. One of the main advantages of LC-MS/MS is its ability to analyze hormones without derivatization (necessary in GC) or the need of hydrolyze the conjugated form.

Due to low level concentration of these compounds in environmental water, it is necessary to apply an extraction and preconcentration method prior to LC analysis. The most used technique of extraction and preconcentration method for liquid samples is the solid phase extraction (SPE) [19-21].

The objective of this study is to develop a rapid and simple procedure of extraction, preconcentration and determination of fifteen steroid hormones (Table 1), based on solid phase extraction and ultra-high performance liquid chromatography tandem mass spectrometry (SPE-UHPLC-MS/MS). The developed method is applied to the identification and quantification of these compounds in wastewater samples obtained from the influents and effluents of two wastewater treatment plants (WWTPs) of Gran Canaria (Spain). They present different methods of wastewater treatments: WWTP 1 presents a traditional method based on activated sludge, while WWTP 2 uses a membrane bioreactor technique.

2. Materials and methods

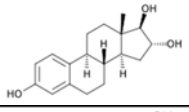
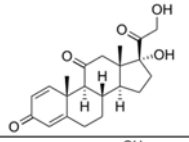
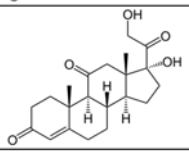
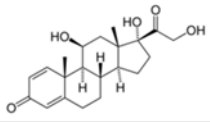
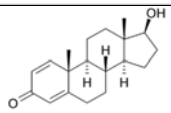
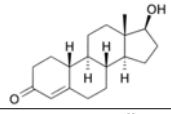
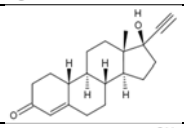
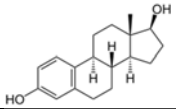
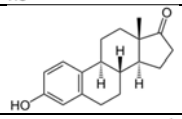
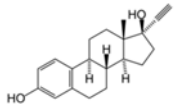
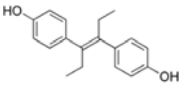
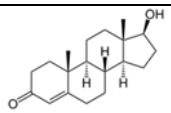
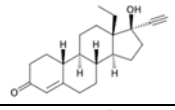
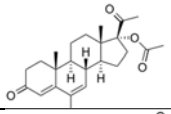
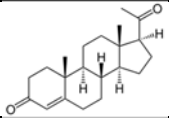
2.1. Reagents

All of the hormonal compounds used 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 4ºC prior to use. Working aqueous standard solutions were prepared daily. Ultrapure water 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 and the ammonium acetate used to adjust the pH of the mobile phases were obtained from Panreac Química (Barcelona, Spain).

2.2. Sample collection

Water samples were collected from the effluents of two wastewater treatment plants located in the northern area of Gran Canaria in May of 2013. WWTP1 used a conventional activated sludge treatment system, while WWTP2 employed a membrane bioreactor treatment system. The samples were collected in 2 L amber glass bottles that were rinsed beforehand with methanol and ultrapure water. Samples were purified through filtration with fibreglass filters and then with 0.65 µm membrane filters (Millipore, Ireland). The samples were stored in the dark at 4°C.

Table 1. List of hormonal compounds, pKa values, chemical structure, and retention times.

Compound		pK_a[25]	Structure	t_R (min)
E3	Estriol	10.3		2.12
PRED	Prednisone	12.4		2.29
COR	Cortisone	12.4		2.32
PREDNL	Prednisolone	12.5		2.45
BOL	Boldenone	15.1		2.87
NAN	Nandrolone	15.1		2.93
NORET	Norethisterone	13.1		2.95
E2	17β-estradiol	10.3		2.97
E1	Estrone	10.3		2.99
EE	17α-ethinylestradiol	10.3		3.00
DES	Diethylstilbestrol	10.2		3.02
TES	Testosterone	15.1		3.15
NOR	Norgestrel	13.1		3.29
MGA	Megestrolacetate	--		3.54
PRO	Progesterone	--		3.71

2.3. Instrumentation

For the SPE optimization, the instrument used was an ultra-high performance liquid chromatography with fluorescence detector (UHPLC-FD) system consisting of an ACQUITY Quaternary Solvent Manager (QSM) used to load samples and wash and recondition the analytical column, an autosampler, a column manager and a fluorescence detector with excitation and emission wavelengths of 280 and 310 nm respectively, all from Waters (Madrid, Spain).

The analysis of wastewater samples was performed in a UHPLC-MS/MS system from Waters (Madrid, Spain), similar to the described above, with a 2777 autosampler equipped with a 25 µL syringe and a tray to hold 2 mL vials, and a ACQUITY tandem triple quadrupole (TQD) mass spectrometer with an electrospray ionization (ESI) interface. All Waters components (Madrid, Spain) were controlled using the MassLynx Mass Spectrometry Software. Electrospray ionisation parameters were fixed 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/hr. Nitrogen was used as the desolvation gas, and argon was employed as the collision gas.

The detailed MS/MS detection parameters for each hormonal compound are presented in Table 2 and were optimised by the direct injection of a 1 $mg \cdot L^{-1}$ standard solution of each analyte into the detector at a flow rate of 10 $\mu L \cdot min^{-1}$.

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

Compound	Precursor ion (m/z)	Capillary voltage (Ion mode)	Quantification ion, m/z (collision potential, V)	Confirmation ion, m/z (collision potential, V)
E3	287.2	-65 V (ESI –)	171.0 (37)	145.2 (39)
PRED	359.3	30 V (ESI +)	147.0 (15)	237.0 (20)
COR	361.3	30 V (ESI +)	163.0 (25)	121.0 (45)
PREDNL	361.3	20 V (ESI +)	147.1 (20)	173.1 (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)
NORET	299.2	30 V (ESI +)	109.1 (25)	91.0 (40)
E2	271.2	-65 V (ESI –)	145.1 (40)	183.1 (31)
E1	269.2	-65 V (ESI –)	145.0 (36)	143.0 (48)
EE	295.2	-60 V (ESI –)	145.0 (37)	158.9 (33)
DES	267.1	-50 V (ESI –)	237.1 (29)	251.1 (25)
TES	289.2	38 V (ESI +)	97.0 (22)	104.0 (21)
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)

2.4. Chromatographic conditions

For the SPE optimization, 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 Chromatography, Barcelona, Spain) operating at a temperature of 30°C. Analytes separation was carried out employing the following gradient: starts at 55:45 (v/v) water:methanol for 1 minute. During 3 minutes, it changed to 50:50 (v/v) and stayed for 2.5 minutes more. Finally, came back to initial conditions in 1 minute, and stayed for 1.5 minutes. Therefore, the analysis took 9 minutes at a flow of 0.5 $mL \cdot min^{-1}$.

For the analysis of real samples, a UHPLC-MS/MS system was used. The analytical column was the same, and the mobile phase was water adjusted with a buffer consisting in 0.1% v/v ammonia, and 15mM of ammonium acetate and methanol. The analysis was performed in gradient mode at a flow rate of 0.3 $mL \cdot min^{-1}$. The gradient started at 80:20 (v/v) mixture of water:methanol, which changed to 40:60 (v/v) in 1.5 minutes, and to 25:75 in 1.25 minutes. Following, stayed at 25:75 for 1 minute and returned to 80:20 in 2.25 minutes. Finally, stayed calibrating for another 0.5 minutes more. The whole analysis took 6.5 minutes. The sample volume injected was 10 µL.

3. Results and discussion

3.1. Solid-Phase Extraction (SPE) optimization

There are a number of parameters that affect to SPE procedure such us type of sorbent, pH, ionic strength, sample and desorption volumes and wash step. To optimize these parameters, it was used Milli-Q water spiked with a solution of fluorescence oestrogens (estriol, 17β-estradiol and 17α-ethinylestradiol) to obtain a final concentration of 250 $\mu g \cdot L^{-1}$.

The first parameter to optimize is the choice of sorbent, since it controls the selectivity, affinity and capacity over analytes. In this study, the SPE cartridges used were OASIS HLB, SepPak C_{18} (both from Waters, Madrid, Spain) and BondElut ENV (from Agilent, Madrid, Spain). From the results obtained, better signals were found for SepPak C_{18} cartridge

After choose the optimum cartridge, we used an initial experimental design of 2^3 (three parameters at two levels), to study the influence of pH (3 and 8), ionic strength (0 and 30% (w/v) of NaCl) and sample volume (50 and 250 mL) over extraction process, obtaining the influence of each parameter and the variable correlation to each other. The experimental design was obtained using Statgraphics Plus software 5.1 and the statistics study was done with IBM SPSS Statistics 19.

It was observed that the ionic strength and sample volume had the major influence on the recoveries of the analytes. For that, a 3^2 factorial design to optimize these two variables at three levels per parameter (0, 15 and 30% (w/v) of NaCl for ionic strength and 50, 100 y 250 mL for sample volume) was used. The results obtained shown that an increment of the ionic strength did not produce an increase in the response area of the compound, and the optimum volume was 250 mL. Finally, the desorption volume (1 mL of methanol and 2 mL of methanol in one and two steps) and wash-step (5

mL of Milli-Q water, and 5 mL of Milli-Q water with 5 and 10% of methanol (v/v)) were assayed to complete the optimization of the SPE process. The optimum values were 2 mL of methanol in one step and 5 mL of Milli-Q water without methanol respectively.

In accordance with the obtained results, the optimum conditions for SPE procedure were: SepPak C_{18} cartridge, 250 mL of sample at pH=8 and 0% of NaCl, desorption with 2 mL of methanol in one step and wash step with 5 mL of Milli-Q water. In these conditions, we achieved a preconcentration factor of 125 [22].

3.2. Analytical parameters

An internal calibration curve was used for the quantification of the analytes by diluting the stock solution of each analyte, into the samples to concentrations ranging between 1 and 100 $\mu g \cdot L^{-1}$. Analysis was conducted by UHPLC-MS/MS and linear calibration plots for each analyte ($r^2 > 0.99$) were obtained based on their chromatographic peak areas. Table 3 shows the analytical parameters obtained for all compounds analysed.

The limit of detection (LOD) and the limit of quantification (LOQ) for each compound were calculated from the signal to noise ratio of each individual peak. The LOD was defined as the lowest concentration that gave a signal to noise ratio that was greater than 3. The LOQ was defined as the lowest concentration that gave a signal to noise ratio that was greater than 10. The LODs ranged from 0.04 – 1.63 $ng \cdot L^{-1}$ and the LOQs ranged from 0.13 – 5.43 $ng \cdot L^{-1}$.

Table 3. Analytical parameters for the SPE-UHPLC-MS/MS method.

Compound	RSD[a] (%)		LOD[b]	LOQ[c]	Recovery (%)	
	(25 $ng \cdot L^{-1}$) n=6	(150 $ng \cdot L^{-1}$) n=6	($ng \cdot L^{-1}$)	($ng \cdot L^{-1}$)	(25 $ng \cdot L^{-1}$) n=3	(150 $ng \cdot L^{-1}$) n=3
E3	5.12	6.50	1.35	4.52	106 ± 8	99 ± 2
PRED	12.9	4.79	0.27	0.91	121 ± 5	105 ± 3
COR	4.55	4.50	0.05	0.16	118 ± 1	103 ± 6
PREDNL	6.90	4.56	0.18	0.58	112 ± 5	99 ± 3
BOL	5.67	4.47	0.17	0.57	117 ± 3	112 ± 10
NAN	3.83	3.81	1.00	3.33	119 ± 4	102 ± 6
NORET	7.08	4.37	0.22	0.74	112 ± 2	99 ± 5
E2	12.4	6.57	1.63	5.43	120 ± 8	103 ± 8
E1	3.51	4.87	0.35	1.18	122 ± 9	109 ± 2
EE	6.84	6.63	2.95	9.83	nq[d]	103 ± 10
DES	9.15	5.79	0.16	0.53	98 ± 14	80 ± 12
TES	6.33	3.82	0.04	0.13	118 ± 3	103 ± 5
NOR	5.14	3.68	0.32	1.06	115 ± 3	103 ± 4
MGA	8.69	4.98	0.22	0.73	107 ± 1	99 ± 3
PRO	7.41	4.59	0.21	0.71	115 ± 4	101 ± 4

[a] Relative Standard Derivation

[b] Detection limits, calculated as signal to noise ratio of three times

[c] Quantification limits, calculated as signal to noise ratio of ten times

[d]not quantified

The performance and reliability of the process were studied by determining the repeatability of the quantification results for all target analytes under the described conditions, using six samples (n=6). The relative standard deviations (RSDs) were lower than 12.9% in all cases, indicating a good repeatability. It was necessary to study the recoveries of all the hormonal compounds, using the optimized SPE-UHPLC-MS/MS method. In table 3 it can be observed that all the compounds under study showed good recoveries, over 80%.

3.3. Matrix effect

Despite the high sensitivity and low chemical noise in UHPLC-MS/MS systems, the sample composition has a great influence on the analyte signal [23]. To evaluate the relative signal enhancement or suppression in the samples the algorithm by Vieno [24] was used.

$$\frac{As - (Asp - Ausp)}{As} x\ 100$$

Where *As* corresponds to the peak area of the analyte in pure standard solution, *Asp* to the peak area in the spiked matrix extract and *Ausp* to the matrix extract. This procedure was applied to an effluent sample, assuming that all matrices will behave in the same way.

For negative ionization compounds, the suppression effects were between -5 and 40%, lower than positive ionization compounds which presented signal suppressions between 60 and 80%. For all compounds, the matrix effect were higher in the samples spiked with 150 ng·L^{-1} than samples spiked with 25 ng·L^{-1}. There were not significant differences between influent and effluent samples. The results obtained are showed in Figure 1.

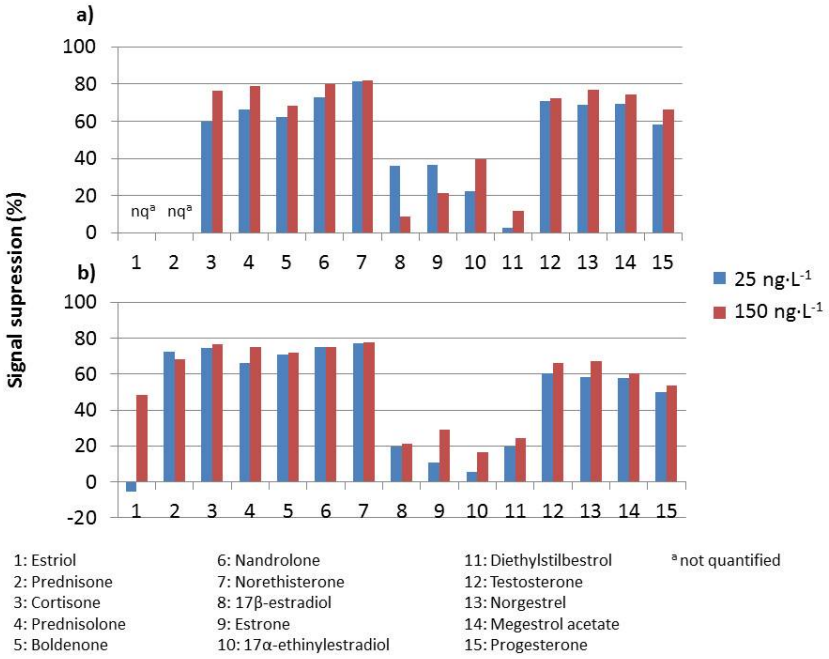


Fig. 1: *Ionization suppression for the hormones studied in (a) influent wastewater, (b) effluent wastewater.*

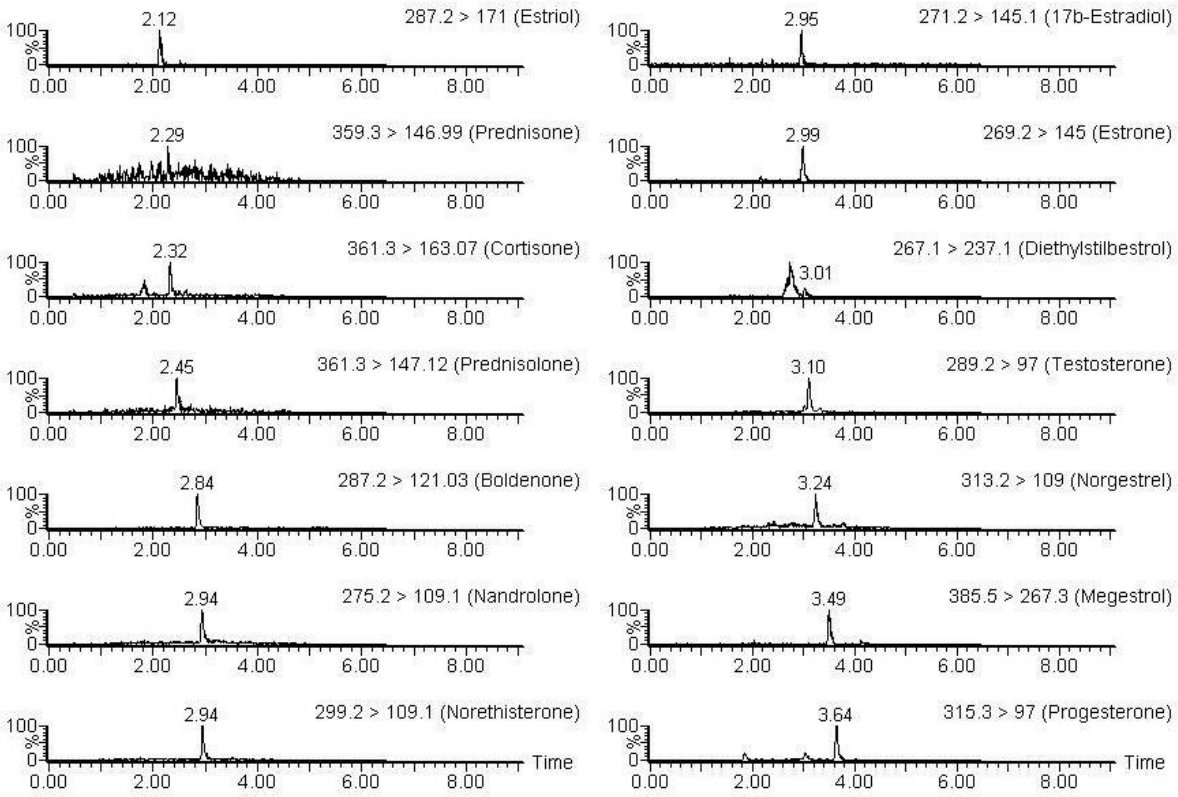


Fig. 2:*Chromatogram of spiked wastewater sample (200 ng·L^{-1} of each analyte) using SPE-UHPLC-MS/MS method*

3.4. Analysis of selected compounds in wastewater samples

To check the efficiency of the developed method, it was applied to determination of target analytes in different wastewater samples from two WWTPs of the island of Gran Canaria (Spain). In figure 2 is shown a chromatogram of spiked wastewater sample (80 ng·L^{-1} of each analyte) using SPE method to extract de analytes. Only influent samples presented concentrations of some compounds, while in effluent samples, the hormones under study were not detected. It can be observed in Table 4 that were detected five hormones (boldenone, norethisterone, 17β-estradiol, estrone and testosterone) in both wastewater treatment plants. The concentrations in both WWTPs ranged from 5.05 to 191.92 ng·L^{-1}.

Table 4. Concentrations of hormones detected in influent samples.

Compound	WWTP 1 (ng·L^{-1})	WWTP 2 (ng·L^{-1})
BOL	29.13 ± 2.74	5.05 ± 0.90
NORET	70.43 ± 0.41	37.03 ± 3.98
E2	nd [a]	40.98 ± 2.30
E1	39.92 ± 5.74	191.92 ± 25.77
TES	18.78 ± 1.10	33.82 ± 1.90

[a] not detected

4. Conclusions

An analytical method for the simultaneous extraction, preconcentration and determination of fifteen hormones (five oestrogens, three androgens, four progestogens and three adrenocortical hormones) in wastewater matrices has been optimized and developed. The method used was solid phase extraction (SPE) for the extraction/preconcentration step and it was combined with UHPLC-MS/MS. The limits of detection reached were between 0.04 - 1.63 $ng \cdot L^{-1}$. In addition, the method presented high recoveries, up to 80%, for the majority of compounds and RSD lower than 13%.

The application of the method to samples from two different WWTPs showed that the concentrations of hormones found, only in influent samples, ranged from 5 to 192 $ng \cdot L^{-1}$.

Acknowledgements

This work was supported by funds provides by the Spanish Ministry of Science and Innovation Research Project CTQ-2010-20554

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