



UNIVERSIDAD DE LAS PALMAS  
DE GRAN CANARIA

## TESIS DOCTORAL

Programa de Doctorado en Gestión Costera

### **OPTIMIZACIÓN Y DESARROLLO DE MÉTODOS DE EXTRACCIÓN PARA LA DETERMINACIÓN DE HORMONAS ESTEROIDEAS. APLICACIÓN EN MUESTRAS DE INTERÉS AMBIENTAL DE LA ISLA DE GRAN CANARIA.**

Optimization and development of extraction methods for the analysis of steroid hormones. Application in environmental samples from Gran Canaria island.

**Rayco Guedes Alonso**

Las Palmas de Gran Canaria, mayo de 2017









**D<sup>a</sup> MARÍA ISABEL PADILLA LEÓN, SECRETARIA DE LA FACULTAD DE CIENCIAS DEL MAR, ÓRGANO RESPONSABLE DEL PROGRAMA DE DOCTORADO EN GESTIÓN COSTERA, DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.**

### CERTIFICA

Que el Consejo de Doctores del Programa de Doctorado en Gestión Costera, en su sesión de fecha 25 de mayo de 2017, tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada: *“Optimización y Desarrollo de Métodos de Extracción para la Determinación de Hormonas Esteroideas. Aplicación en Muestras de Interés Ambiental de la Isla de Gran Canaria”*, presentada por el doctorando: **D. Rayco Iván Guedes Alonso** y dirigida por los Doctores D. José Juan Santana Rodríguez y D<sup>a</sup> Zoraida Sosa Ferrera.

Asimismo, se acordó el informar favorablemente la solicitud para optar a la Mención Internacional del Título de Doctor, por cumplir los requisitos reglamentarios.

Y para que así conste, a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, tribunal defensa y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo el presente en Las Palmas de Gran Canaria, a veinticinco de mayo de dos mil diecisiete.

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Departamento de Química  
Programa de Doctorado en Gestión Costera

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## Resumen

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Durante su existencia, el ser humano ha creado un equilibrio con el medio ambiente que se ha ido volviendo más y más frágil con el paso de los siglos y el desarrollo de las civilizaciones. Este equilibrio se ha visto alterado debido a la explotación indiscriminada de los recursos naturales, lo que ha producido un deterioro progresivo del medio ambiente. A partir de la Revolución Industrial, la producción de productos químicos, tanto orgánicos como inorgánicos, comienza a sufrir un crecimiento exponencial debido al desarrollo del mundo moderno, lo cual convierte al medio ambiente en medio receptor de los desechos generados por las actividades humanas. Este impacto ha sido más acusado en las inmediaciones de los ecosistemas acuáticos a pesar, incluso, de la gran capacidad amortiguadora de dichos ecosistemas, debido a que tradicionalmente el ser humano se ha desarrollado en lugares cercanos a fuentes de agua.

## *Resumen*

Especialmente preocupantes son los nuevos "contaminantes emergentes" (ECs), término que se utiliza para abarcar tanto compuestos recién desarrollados, así como compuestos que recientemente han sido categorizados como contaminantes. Estos compuestos constituyen un importante y heterogéneo grupo de contaminantes y han despertado la preocupación de la comunidad científica internacional debido a hechos como que pueden ser considerados pseudo-persistentes por su continua entrada en el medio, a que presentan efectos nocivos que en muchos casos se desconocen, y a que pueden ser altamente tóxicos para los organismos de los ecosistemas a los que llegan tras ser utilizados en las diferentes actividades humanas.

Existen multitud de tipos de contaminantes emergentes, como los productos de cuidado personal, los fármacos, algunos pesticidas, los retardantes de llama, los surfactantes, y otros compuestos químicos sin los cuales la vida, tal y como la conocemos en la actualidad, no sería posible.

Dentro de estos contaminantes emergentes, las hormonas esteroideas conforman un grupo de compuestos, de naturaleza tanto natural como antropogénica, que puede dividirse en hormonas sexuales y glucocorticoides. Este grupo de contaminantes emergentes despierta un gran interés debido al amplio uso que tienen en nuestra vida diaria, razón por la cual presentan una introducción continua en el medio. Por ello, y a pesar de que se encuentran en éste a niveles traza, es necesario un estudio en profundidad que permita conocer su presencia, ya que pueden producir diferentes efectos sobre el medio, incluso a concentraciones de una parte por trillón.

La determinación de hormonas a estos niveles de concentración se convierte en un verdadero reto para el químico analítico, pues es necesario el desarrollo de metodologías de extracción y preconcentración que permitan extraer las hormonas de la matriz en la que se encuentran y preconcentrarlas hasta llegar a unos niveles en los que puedan ser medidas con eficacia. Además, para conseguir un análisis sensible y selectivo, se hace necesario el desarrollo de metodologías de separación y detección que permitan la determinación precisa de las hormonas esteroideas a las concentraciones en las que se encuentran en el medio.

En este sentido, la presente Tesis Doctoral se centra en la optimización y desarrollo de metodologías analíticas que permitan la extracción, preconcentración, separación y determinación de estas hormonas en muestras medioambientales de la isla de Gran Canaria tanto de naturaleza líquida, como sólida y biológica.

La realización de esta Tesis Doctoral ha permitido la determinación de la presencia de hormonas esteroideas en muestras de agua procedentes de diversas estaciones depuradoras de aguas residuales, en lodos obtenidos en estas mismas estaciones y en organismos marinos cuyo hábitat se encuentra en las proximidades de los emisarios submarinos utilizados por las estaciones depuradoras para verter las aguas residuales regeneradas. Los resultados obtenidos han permitido, además, evaluar de manera integral el impacto de las hormonas esteroideas en el medio marino de Gran Canaria.



## Abstract

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Throughout history, the human race has built an ecological balance that has become more and more fragile because of the passing of time and the development of civilizations. This balance has been altered due to the indiscriminate exploitation of natural resources, resulting in a progressive deterioration of the Environment. Since the Industrial Revolution, the production of organic and inorganic chemicals has grown exponentially as a result of the development of the modern world, and that turned the Environment into a recipient of the human activities wastes. The environmental footprint is more pronounced in the vicinity of the aquatic ecosystems even though these water resources have a high buffer capacity because the tendency of creating human settlements next to hydrological resources.

There is an important concern about new contaminants called “emerging pollutants” (EPs). This term is used to refer both compounds

## *Abstract*

recently developed and chemical compounds recently classified as pollutants. Emerging pollutants consist in a wide and heterogeneous group of chemicals which have caused an important international scientific concern. This concern is linked to the fact that emerging pollutants are considered as pseudo-persistent pollutants because they are continuously discharged into the environment as well as they have unknown harmful effects and can be toxic to the ecosystem organisms they reach after being used in the different human activities.

There are many kinds of emerging pollutants, such as personal care products, pharmaceutical compounds, some pesticides, flame retardants, surfactants, or industrial compounds. Without them, life as known today would not be possible.

Among emerging pollutants, steroid hormones are a group of natural and anthropogenic compounds which can be divided into sex hormones and glucocorticoids. This group of compounds has attracted the attention of the scientific society because hormones are used in our daily life which produce a continuous discharge into the aquatic ecosystems. Concentrations of steroid hormones in the Environment are in trace levels but it is necessary a deep study which allows to know real and accurate environmental concentrations of steroid hormones, because they could produce harmful effects over aquatic biota, even at part-per-trillion concentrations.

The determination of hormones in these concentration levels are a genuine challenge for analytical chemists because it is necessary the development of extraction and preconcentration methodologies which permit the extraction of hormones from the matrix where they are

present and, after that, preconcentrate them to measurable levels in order to quantify them properly. Moreover, it is vital the development of separation and detection technologies, which allow selective and sensitive analysis of steroid hormones at that trace concentrations.

This Doctoral Thesis is focused in the study of analytical methodologies which permit the extraction, preconcentration, separation and determination of a group of emerging pollutants consisting of steroid hormones in environmental liquid, solid and biological samples of Gran Canaria Island.

The fulfilment of this Doctoral Thesis have made possible the determination of the presence of steroid hormones in wastewater samples from different wastewater treatment plants, in sludge of these plants and in marine organisms exposed to submarine outfalls used by wastewater treatment plants to spill purified waters. The obtained results have been used to evaluate the impact of steroid hormones in the aquatic ecosystem of Gran Canaria Island.



# Capítulo 1. Introducción

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El desarrollo del mundo moderno ha estado íntimamente ligado con la utilización de una gran cantidad de productos químicos de diferente naturaleza para diferentes propósitos, lo que ha traído consigo un deterioro del medio ambiente debido a que éste se ha convertido en el recipiente de todos los residuos generados por el ser humano y sus actividades. Cada día llegan al medio ambiente toneladas de compuestos, tanto orgánicos como inorgánicos, cuyos efectos sobre él pueden persistir décadas, o incluso siglos. Afortunadamente, la capacidad de regeneración del medio ambiente ha hecho que esta situación no sea insostenible en pocos años, pero esta capacidad es, en muchos casos, limitada, por lo que las concentraciones que van quedando retenidas en los diferentes compartimentos medioambientales son cada vez mayores. Éste, entre otros muchos fenómenos, ha llevado a que en las últimas décadas se incremente cada vez más la sensibilización sobre el medio ambiente y la

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necesidad de que el ser humano lleve a cabo un desarrollo sostenible, que le permita vivir en las mismas condiciones que en la actualidad, sin que se produzca una degradación del medio que lo haga inhabitable a medio o largo plazo.

En este sentido, la química analítica ha afrontado el reto de desarrollar metodologías analíticas que permitan la medición correcta, sensible y reproducible de las concentraciones de contaminantes que se encuentran en el medio, así como en las fuentes que la producen. Por ello se habla de análisis de presencia de contaminantes y de análisis del transporte de éstos entre los diferentes compartimentos medioambientales. A la hora de desarrollar estos métodos analíticos existen multitud de variables que deben ser tenidas en cuenta como son el tipo de compuesto que se quiere analizar, la matriz en la que éstos se encuentran, el proceso de extracción del contaminante, así como el método de determinación más adecuado o los reactivos necesarios que produzcan el menor impacto posible sobre el medio ambiente. Por todo ello, las investigaciones en el campo de la química analítica de las últimas décadas se han centrado en el desarrollo de metodologías cada vez más sensibles y selectivas que permitan la determinación de dichos contaminantes. Este desarrollo está actualmente en pleno apogeo debido a las tendencias actuales de miniaturización, automatización y desarrollo de nuevos materiales y técnicas.

De la variedad de contaminantes que llegan a diario al medio ambiente se puede hacer una primera distinción entre contaminantes inorgánicos y orgánicos. La subdivisión de estos dos grandes grupos, sobre todo de los contaminantes orgánicos, se antoja bastante más complicada debido a la gran cantidad de compuestos existentes, los cuales presentan

diferentes estructuras y propiedades químicas, así como variados efectos sobre el medio. Algunas regulaciones, como por ejemplo la Directiva Europea 2000/60/CE (Directiva Europea Marco del Agua), por la que se establece un marco comunitario de actuación en el ámbito de la política de aguas, hacen una distinción entre tipos de contaminantes [1]. Dicha normativa diferencia entre sustancias prioritarias y contaminantes emergentes, siendo la principal diferencia entre estos grupos el grado de conocimiento que se tiene sobre ellos en cuanto a su toxicidad sobre organismos acuáticos, su persistencia en el medio acuático y su capacidad de ser degradados. Por esta razón es necesaria una investigación constante sobre los contaminantes que llegan al medio, incluso a concentraciones bajas, pues sus efectos nocivos pueden producirse incluso a concentraciones de nanogramos por litro [2]. Esta investigación constante, tanto de las características y efectos adversos de los compuestos orgánicos como de nuevas técnicas de mayor selectividad, sensibilidad y reproducibilidad, permitirá mantener vivas estas reglamentaciones y, por tanto, un medio ambiente más protegido y diverso. Teniendo como base la diferenciación que realiza la Directiva Europea Marco del Agua, se hace necesaria la investigación en lo referente a los contaminantes emergentes, por ser éstos de los que se dispone menos información y como consecuencia, presentan una toxicidad potencial mayor.

### 1.1. Los contaminantes emergentes

Los contaminantes emergentes constituyen por sí mismos un importante grupo de compuestos cuyo número está en continuo crecimiento debido a que incluye todos aquellos compuestos orgánicos

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que pueden producir un efecto adverso sobre el medio, es decir, pueden producir contaminación. Esta contaminación, en muchos casos, no es reciente, pues los compuestos han sido utilizados tanto en el ámbito doméstico como en el industrial durante décadas, presentando una gran ubicuidad. Sin embargo, las bajas concentraciones a las que se encuentran en el medio han pasado desapercibidas para una sociedad científica centrada en el control y estudio de aquellos contaminantes recogidos en las legislaciones y de los que se tenía un amplio conocimiento sobre sus efectos nocivos en organismos. De hecho, no es hasta finales de los años 90 cuando empieza a existir una concienciación científica acerca de los contaminantes emergentes.

En la Figura 1 se muestra la evolución en el número de artículos científicos en cuyo título o resumen aparecen las palabras “*emerging pollutants*”, pudiéndose comprobar que el número de artículos y, por tanto, de investigaciones, ha aumentado constantemente, lo cual refleja el interés de la comunidad científica por este incipiente problema medioambiental.

El hecho de crear una categoría de nuevos contaminantes surgió, por tanto, hace pocos años, a raíz de la mejora de los métodos de análisis químico, con los cuales se pudo comprobar que los diferentes compartimentos medioambientales presentaban concentraciones de “nuevos” compuestos químicos que más tarde se catalogaron como peligrosos o tóxicos para el medio acuático. Esto hizo que algunos contaminantes emergentes pasaran a la lista de sustancias prioritarias, como es el caso de detergentes basados en moléculas de octil o nonilfenol, los difeniléteres bromados utilizados como retardantes de llama o los hexabromociclododecanos [1]. Además, una de las razones por

las que estos compuestos han sido considerados sustancias prioritarias es que se ha descubierto que, además de ser tóxicos para el medio, pueden producir cambios en el desarrollo, comportamiento o reproducción de organismos vivos por ser compuestos que pueden comportarse como hormonas.

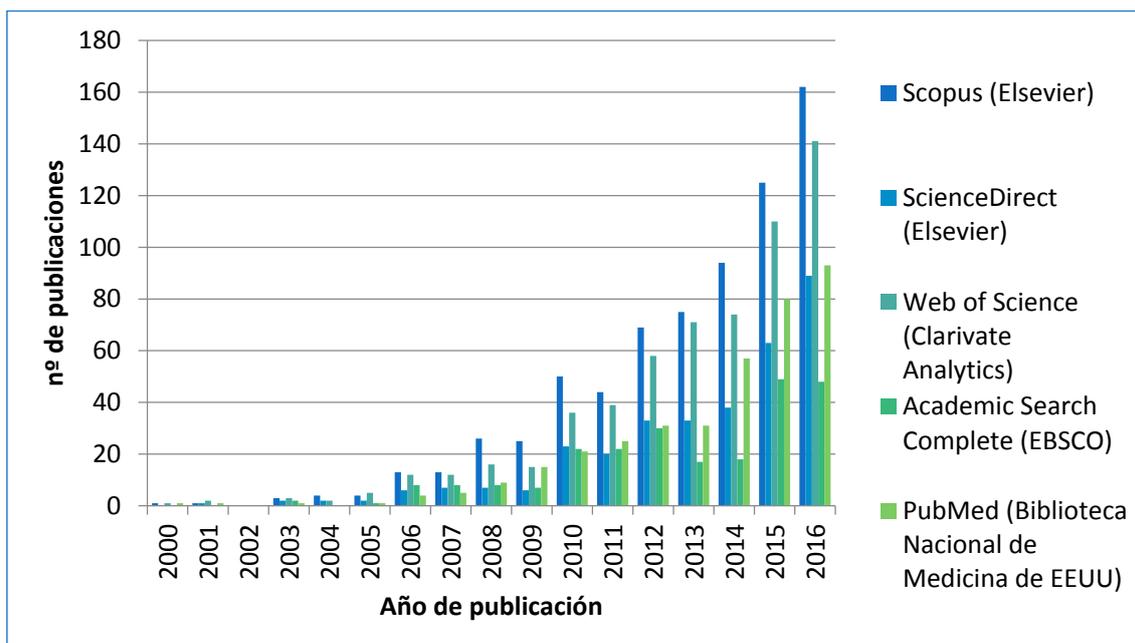


Figura 1.1. Publicaciones en revistas científicas cuyo título o resumen incluye las palabras “emerging pollutants” en diferentes bases de datos bibliográficas

Sin embargo, el paso de contaminantes emergentes a listas de sustancias prioritarias es más lento que el crecimiento por sí mismo de la lista de contaminantes emergentes. Por ejemplo, en los últimos años ha aumentado el interés acerca de los fármacos y los productos de cuidado personal, conocidos como PPCPs por sus siglas en inglés (*pharmaceuticals and personal care products*), los cuales constituyen un importante grupo dentro de los contaminantes emergentes ya que, por sus características, es muy probable que puedan producir un impacto negativo cuando lleguen al medio ambiente. Sin embargo, el conocimiento acerca de la

## Capítulo 1. Introducción

ecotoxicidad de los PPCPs sobre el medio ambiente es aún limitado, lo cual se une a problemas como una falta de información detallada sobre su presencia en éste, así como la heterogeneidad en cuanto a propiedades y usos, lo que hace que la regulación por parte de los organismos de control sea complicada y la reglamentación, escasa.

Estas razones han provocado que la investigación acerca de los contaminantes emergentes sea considerada prioritaria para diversos organismos internacionales de salud pública y cuidado del medio ambiente como la Agencia de Protección del Medio Ambiente de Estados Unidos (US-EPA), la Organización Mundial de la Salud (OMS) o la Comisión Europea (CE) [3]. Formalmente, la CE define a los contaminantes emergentes como *“contaminantes que en la actualidad no están incluidos en los programas de seguimiento sistemático en la Unión, pero que suponen un importante riesgo, lo cual exige su regulación, dependiendo de sus posibles efectos ecotoxicológicos y toxicológicos, y de sus niveles en el medio acuático”* [1].

El principal interés de los organismos internacionales es conocer pormenorizadamente las características, efectos y transporte de los contaminantes emergentes en el medio ambiente para poder realizar reglamentaciones adecuadas a los diferentes compuestos existentes en los distintos compartimentos ambientales. Sin embargo, la tarea de recabar datos sobre todos los compuestos químicos existentes en una tarea prácticamente imposible ya que existen cientos, o incluso miles, de compuestos considerados como contaminantes emergentes. Esto ha hecho que las estrategias de recogida de información se limiten a contaminantes emergentes de los que se tiene una información algo más amplia y de los que se conoce su ecotoxicidad y sus efectos en el medio,

principalmente acuático. En este sentido, algunas organizaciones como la Comisión Europea han elaborado listas de vigilancia de compuestos (denominadas *Watch Lists* en inglés), las cuales recogen un máximo de 10 compuestos o familias de compuestos similares que deben ser vigilados y de los que se deben recabar la mayor cantidad de datos posibles sobre su presencia, efectos y propiedades [4]. Este estudio detallado sobre un número limitado de compuestos químicos permitiría, en un lapso de tiempo determinado, decidir si constituyen una amenaza real para el medio y, por tanto, si deben pasar a ser considerados como sustancias prioritarias, así como focalizar la investigación y optimizar los recursos.

Debido al carácter eminentemente antropogénico de los contaminantes emergentes, las fuentes de emisión de éstos al medio hay que buscarlas en las fuentes de desechos humanas. Estas fuentes pueden ser puntuales, cuando se conoce el lugar donde se produce el vertido, o difusas, más difíciles de detectar y controlar y normalmente ligadas a actividades humanas como la agricultura y la ganadería [5]. De entre las fuentes puntuales, las estaciones depuradoras de aguas residuales (EDARs) se consideran la fuente principal de contaminantes emergentes al medio acuático [6] ya que a ellas llegan todo tipo de aguas residuales, eminentemente urbanas, pero también aguas de procedencia industrial, agrícola o especial (efluentes de zonas hospitalarias, veterinarias, vertederos, etc.). La finalidad de las estaciones depuradoras de aguas residuales es llevar a cabo una serie de tratamientos, que pueden ser físicos, químicos, biológicos o combinación de éstos, para eliminar tanto la materia suspendida en el agua como la materia orgánica presente en ella. Una vez las aguas han sido tratadas y cumplen con los valores de calidad impuestos por las diferentes administraciones públicas, pueden verterse

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en el medio receptor. Sin embargo, y, debido en gran manera a la falta de legislación, en los efluentes de estas EDARs se pueden detectar concentraciones de contaminantes emergentes debido a que las plantas depuradoras no están diseñadas para eliminarlos ni se tiene la obligación legal de hacerlo. Aparte de dicha fuente, otras vías puntuales de entrada de contaminantes emergentes al medio ambiente pueden ser los vertidos accidentales, las instalaciones agrícolas regadas con aguas depuradas o los lixiviados de vertederos. En cuanto a las fuentes de contaminación difusas se encuentran las fugas de los sistemas de saneamiento, las escorrentías procedentes de instalaciones agrícolas y ganaderas o las infiltraciones de agua de río contaminada en acuíferos subterráneos [5,7,8].

Como se ha expuesto anteriormente, los contaminantes emergentes constituyen un grupo heterogéneo de compuestos orgánicos, los cuales se pueden clasificar, por ejemplo, según el uso que tengan, de manera que se pueden encontrar: fármacos, productos de cuidado personal, drogas de abuso, compuestos utilizados en industria, pesticidas o compuestos perfluorados [3,5]. No obstante, esta clasificación no es tan útil si, como prefieren otros autores, la categorización se realiza según sus características físico-químicas y efectos [9], encontrándose: compuestos persistentes, bioacumulativos y tóxicos (PBTs, *persistent, bioaccumulative and toxic substances*) compuestos polares (fármacos, pesticidas y compuestos usados en actividades industriales), compuestos inorgánicos o disruptores endocrinos (EDCs, *endocrine disruptor compounds*). Esta última familia de compuestos, ha recibido una gran consideración en los últimos años debido principalmente al gran impacto que pueden tener sobre los organismos acuáticos, por lo que se tratarán con mayor profundidad en el siguiente apartado.

### 1.2. Los compuestos disruptores endocrinos

Tal y como se ha expuesto anteriormente, los compuestos disruptores endocrinos constituyen una subcategoría dentro de los contaminantes emergentes que ha cobrado una gran relevancia en los últimos años debido a su potencial efecto nocivo para los organismos. Sin embargo, antes de estudiar en profundidad este tipo de compuestos es necesario conocer el sistema en el que se producen la mayor parte de sus efectos.

#### 1.2.1. El sistema endocrino

El sistema endocrino, también denominado sistema hormonal, es un sistema de vital importancia para los vertebrados debido a que, junto al sistema nervioso, controla la actividad de los distintos órganos. La función principal del sistema endocrino es el control de aquellos procesos “lentos” que tienen lugar en el organismo, como pueden ser el metabolismo, el crecimiento o los procesos reproductivos. Es por ello por lo que una alteración en este sistema puede producir cambios en el organismo que no se detecten rápidamente y que además éstos sean duraderos en el tiempo o incluso irreversibles.

El sistema endocrino está formado por glándulas, hormonas y células receptoras y el equilibrio entre estas partes es vital para el correcto funcionamiento del sistema. En primer lugar, encontramos las glándulas, que son aquellos órganos encargados de segregar las diferentes hormonas que necesitará el organismo. En el ser humano existen diversas glándulas, las cuales se muestran en la figura siguiente extraída del trabajo de Schug y colaboradores [10].

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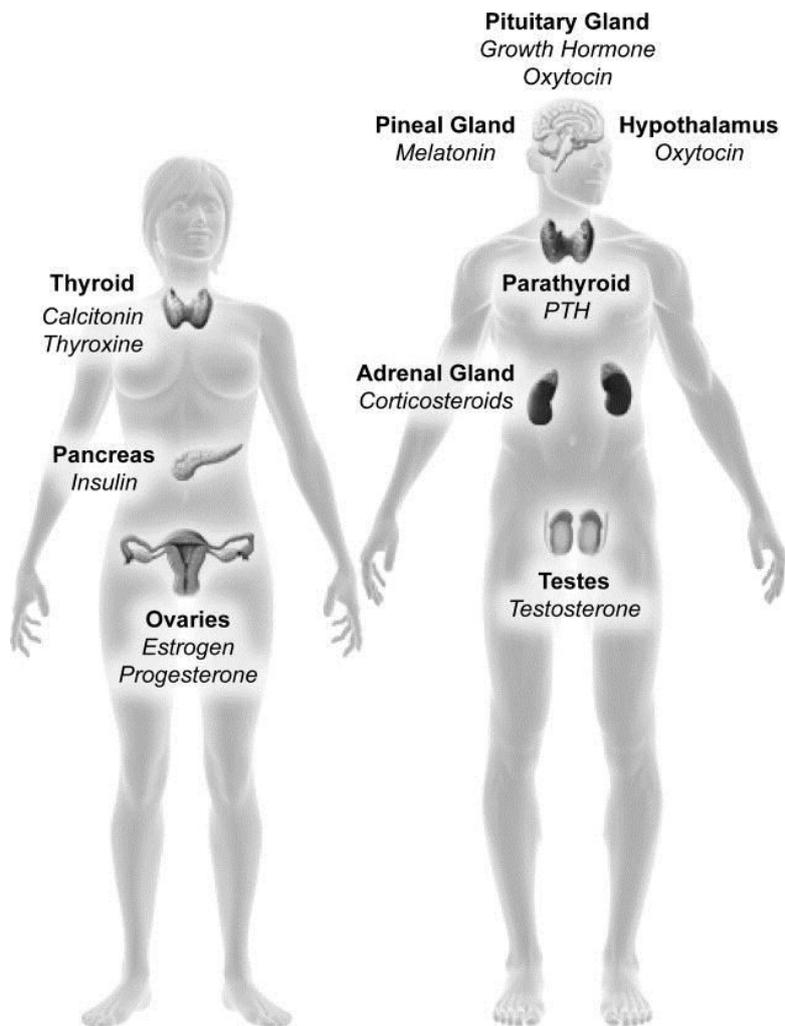


Figura 1.2. Glándulas del sistema endocrino del ser humano y sus respectivas hormonas segregadas.

Todas estas glándulas segregan las diferentes hormonas que serán las encargadas de viajar por el torrente sanguíneo para llegar a las células receptoras. Estas células receptoras o diana se encuentran en los diferentes órganos y tejidos y cuentan con un receptor hormonal capaz de reconocer la hormona y desencadenar la reacción que ésta les indica. Este proceso es el que se conoce como efecto endocrino. Sin embargo, en ocasiones las hormonas no tienen que viajar por el torrente sanguíneo hasta un órgano en cuestión. En estos casos hablamos de efecto paracrino, cuando las células receptoras están adyacentes a las células

glandulares que producen las hormonas o de efecto autocrino cuando la producción de hormonas y recepción de ésta ocurre en la misma célula [11].

Existen diferentes tipos de hormonas, entre las que encontramos péptidos de diferentes tamaños, esteroides, derivados del colesterol o aminoácidos y sus derivados [12]. Sin embargo, pese a sus diferentes estructuras y funciones, los diferentes sistemas hormonales se encuentran correlacionados, presentando además una relación jerarquizada [11], por lo que un cambio o mal funcionamiento en cualquiera de estos sistemas endocrinos puede provocar una reacción en cadena que afecte al resto y que se alteren las actividades biológicas que están relacionadas con las hormonas. Estos cambios pueden estar provocados por desórdenes endocrinos ligados a factores incontrolables como el envejecimiento o ciertas enfermedades [13]. Sin embargo, en los últimos quince años se han relacionado, aunque con cierta controversia dentro de la comunidad científica, el efecto de ciertos compuestos químicos con desórdenes hormonales [10,14–16].

### **1.2.2. Definición y tipos de compuestos disruptores endocrinos**

Al igual que ocurre con los contaminantes emergentes, el interés científico acerca de los disruptores endocrinos, sus características, presencia en el medio y efectos es algo nuevo. De hecho, la investigación acerca de este tipo de compuestos se ha desarrollado en los últimos 20 años, por lo que no existe aún un consenso en lo que se refiere a definiciones y clasificaciones.

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El término disruptor endocrino lo utilizó por primera vez Theodora Colman en 1991, fundadora de la organización *The Endocrine Disruption Exchange* (TEDX) [17], la única organización enfocada en los problemas medioambientales y de salud humana producidos por la exposición ambiental o en bajas dosis a compuestos disruptores endocrinos [18]. Sin embargo, más de 20 años después no existe una definición definitiva para este tipo de contaminantes emergentes. La falta de consenso llega incluso a la denominación de este tipo de compuestos, ya que hay autores que los denominan “compuestos disruptores endocrinos, CDEs” (en inglés, *endocrine disrupting compounds*, EDCs), otros prefieren la denominación “contaminante” en vez de “compuesto”, otros, como la Comisión Europea, prefieren denominarlos “alteradores endocrinos” (aunque en inglés se hable de *endocrine-disrupting chemical substances*), mientras que otros simplemente utilizan la denominación acuñada por Colborn de “disruptores endocrinos, DEs” (en inglés, *endocrine disruptors*, EDs).

Por otra parte, en cuanto a la definición de lo que es un compuesto disruptor endocrino, existen puntos en común en todas las definiciones, aunque ésta tampoco se encuentra completamente consensuada, ni dentro de la comunidad científica, ni en las diferentes regulaciones que existen al respecto:

- La primera definición fue del grupo de expertos *Wingspread*, liderado por Theodora Colborn, la cual establecía que los “disruptores endocrinos son sustancias introducidas en el medio ambiente por la actividad humana, capaces de afectar al sistema endocrino de animales, peces incluidos, fauna salvaje y humanos” [19].

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- Según la Comisión Europea en uno de sus más recientes documentos [20], *“Las sustancias químicas que alteran los procesos endocrinos («alteradores endocrinos») son aquellas que perturban las funciones del sistema hormonal y, en consecuencia, producen efectos adversos”*.
- Por su parte, la Organización Mundial de la Salud recoge en su Programa Internacional sobre Seguridad Química [21] que *“un disruptor endocrino es una sustancia exógena o combinaciones de varias que alteran las funciones del sistema endocrino y, por lo tanto, tienen efectos perjudiciales para la salud en un organismo intacto o su progenie, o para determinadas (sub)poblaciones”*.
- La Agencia Estadounidense de protección del Medio Ambiente se ha basado en la definición de Crisp y colaboradores [22] que establece que *“un disruptor endocrino es un agente exógeno que interfiere en la síntesis, secreción, transporte, unión, acción o eliminación de las hormonas naturales que son responsables del mantenimiento de la homeostasis, reproducción, desarrollo y/o comportamiento”*.
- En lo que respecta a las tendencias actuales, algunos grupos de científicos han decidido aceptar la definición establecida por la OMS, pero con algunas ampliaciones en cuanto a *“las alteraciones del sistema endocrino”* los *“efectos adversos”* o a qué se le considera como *“organismo intacto”* [23].

No obstante, existen puntos de encuentro entre todas estas definiciones, como pueden ser la consideración de disruptor endocrino como un compuesto exógeno, que debe provocar cambios en el sistema

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endocrino del organismo al que afecta y que estos cambios producen efectos negativos sobre el organismo o su descendencia, especialmente en etapas clave como pueden ser el crecimiento, la gestación o la lactancia. Además, también existe cierto consenso sobre la clasificación de los disruptores endocrinos, los cuales se pueden dividir, teniendo en cuenta su origen, en compuestos naturales y artificiales o antropogénicos [24] o según su duración en el medio ambiente, en compuestos persistentes o de vida corta [25].

Dentro de los compuestos disruptores endocrinos se encuentran los fitoestrógenos, presentes en multitud de plantas y vegetales consumidos a diario, que presentan una actividad disruptora relativamente débil en comparación con las hormonas endógenas. Por otra parte, los compuestos disruptores endocrinos de carácter antropogénico son bastante más numerosos y se usan en multitud de aspectos de la vida moderna. Se consideran disruptores endocrinos compuestos industriales como plastificantes o plásticos, materiales de empaquetado, pinturas, compuestos ligados a la agricultura como los pesticidas, incluso compuestos presentes en productos farmacéuticos o de cuidado personal. Al igual que ocurre con la inexistencia de una definición estándar para los disruptores endocrinos, no existe tampoco una estructura química que permita determinar que una determinada sustancia es un alterador endocrino. De esta manera, encontramos que algunos compuestos disruptores endocrinos presentan estructuras similares a las hormonas, lo que hace que puedan mimetizar a éstas en los receptores celulares correspondientes. Sin embargo, otros compuestos disruptores, son capaces de alterar el sistema endocrino teniendo estructuras totalmente diferentes a las hormonas a las que mimetizan. En la siguiente tabla se

recogen diferentes tipos de disruptores endocrinos, así como ejemplos de cada uno de estos tipos:

Tabla 1.1. Tipos de compuestos disruptores endocrinos y ejemplos

Compuestos naturales	Fitoestrógenos	Cumestanos (cumestrol) Flavonoides (apigenina, kaempferol, naringenina) Isoflavonoides (genisteína, daidzeína) Lignanos (enterodiol, enterolactona)
Compuestos antropogénicos	Fármacos	Andrógenos Corticoides Diethylstilbestrol Estrógenos y progestágenos
	Metales pesados	Arsénico Cadmio Mercurio Plomo Uranio
	Pesticidas	Atrazina Dicloro difenil tricloroetano (DDT) y metabolitos Dieldrin Diurón Lindano Malatión Permetrina
	Plásticos y plastificantes	Ftalatos (BBP, DBP, DEHP) Bisfenol-A Bifenilos policlorados (PCBs)
	Productos de cuidado personal	Benzofenonas Etilenglicol Parabenos Resorcinol
	Surfactantes	Nonilfenol y derivados Octilfenol y derivados
	Productos industriales	Bifenilos policlorados (PCBs) o polibromados (PBBs) Estireno Diclorometano Tributilestano (TBT) y derivados

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### 1.2.3. Mecanismo de acción y efectos de los compuestos disruptores endocrinos

Se ha podido comprobar que existe una gran variedad de compuestos químicos considerados como contaminantes emergentes, pero, sin embargo, no todos afectan de igual manera al sistema endocrino ya que presentan mecanismos de acción diferentes entre ellos. De hecho, la actividad sobre el sistema endocrino de algunos compuestos químicos se conoce desde mucho antes de la creación del término “disruptor endocrino”, ya que las diferentes investigaciones, así como los protocolos de las diferentes organizaciones gubernamentales, se han centrado más en los efectos producidos sobre el organismo en su totalidad que en los mecanismos de acción a nivel celular o de los tejidos [26]. Ya en los años 30 y 40 se relacionó actividad estrogénica con compuestos con anillos condensados o con hidrocarburos [27,28] y, a partir de estudios realizados en las siguientes décadas, en los cuales se comenzaron a relacionar efectos de compuestos sintéticos similares a estrógenos como el 17 $\beta$ -estradiol o andrógenos como la testosterona, se desarrolló el estudio de los mecanismos de acción de los disruptores endocrinos [29].

Aunque la variedad de compuestos disruptores endocrinos existentes complica la identificación de mecanismos de disrupción de estos contaminantes, los efectos que pueden producir en las diferentes células diana o receptoras son los mismos [30,31]. Tal y como muestra la figura siguiente, un disruptor endocrino puede bloquear la reacción de la célula correspondiente, impidiendo que la hormona encargada de activar la reacción química pueda emplazarse en el receptor celular. Esto, unido a que el compuesto disruptor endocrino no posee la información necesaria para desencadenar la reacción, provoca que ésta no se produzca. Por otra

parte, existen compuestos disruptores endocrinos que poseen estructuras similares a las hormonas que mimetizan, por lo que, en este caso, sí que se desencadenaría la reacción aparejada a la hormona mimetizada. Sin embargo, lo usual es que la reacción química se vea afectada, tanto de manera insuficiente como excesiva, lo cual puede ser igualmente perjudicial para el organismo afectado. Finalmente, existe la posibilidad de que el contaminante disruptor produzca la síntesis de la hormona o del receptor celular, produciendo también un cambio en el funcionamiento normal del sistema.

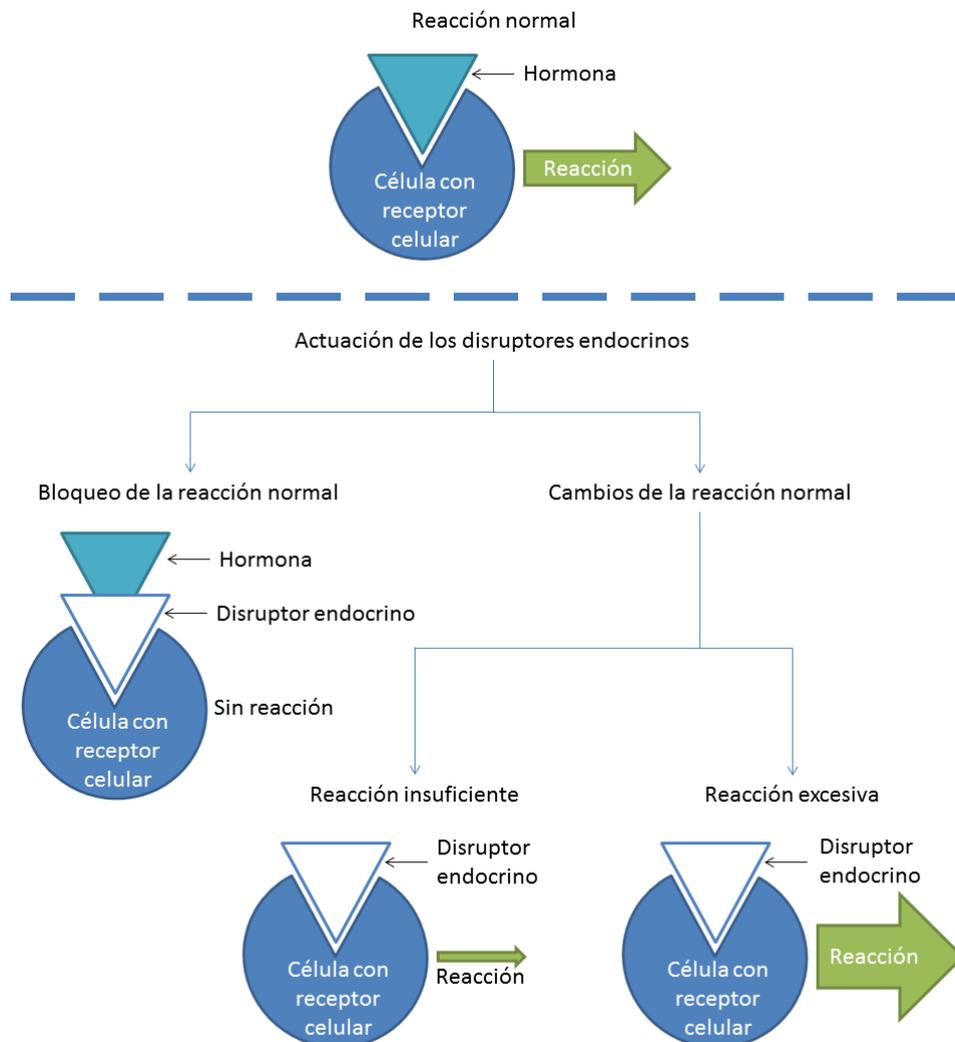


Figura 1.3. Posibles acciones producidas por un compuesto disruptor endocrino al interactuar con una célula receptora. Figura adaptada de [32]

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En cuanto a los mecanismos de acción de los disruptores endocrinos, existe una gran variedad de ellos, que en muchos de los casos no se presentan de manera aislada, sino conjunta [33]. Así pues, los compuestos disruptores endocrinos presentan mecanismos de acción de (anti)estrogenicidad y (anti)androgenicidad, alteración de los diferentes receptores celulares (receptores estrogénicos, androgénicos o de progesterona, receptores estrogénicos asociados a membrana, receptores X retinoides o receptores de aril hidrocarbano), alteración tiroidea o de las células pancreáticas, alteración de las rutas del ácido retinoico y de la vitamina D, inhibición de la producción hormonal o alteración de tejidos diana en el sistema reproductor, cerebro y sistema cardiovascular [33,34]. Además de presentarse en muchas ocasiones de manera conjunta, estos efectos a nivel celular o tisular son dependientes de la concentración a la que el compuesto disruptor se encuentre en el organismo. De esta manera, compuestos tóxicos como las dioxinas, que son mortales a concentraciones significativas, a las concentraciones a las que normalmente se encuentran en el medio pueden producir problemas de reproducción así como efectos estrogénicos o androgénicos [35], dependiendo de las condiciones del organismo y del medio. Por su parte, otros estudios han concluido que dependiendo de la concentración del contaminante, los efectos serán unos o los contrarios, los cuales van desde efectos hormonales positivos o negativos hasta cambios opuestos en la morfología o la fisiología del individuo [33,36,37].

Aunque los efectos a nivel celular, así como los mecanismos de acción, sean comunes para la mayoría de los organismos expuestos a disruptores endocrinos, los efectos generales sobre éstos son variados y

dependen de multitud de factores. Estos factores pueden ser el tiempo de exposición al disruptor, la morfología del organismo, la edad y sexo de éste o, incluso, la presencia de otras hormonas en el organismo u órgano afectado. Por esta razón, en la actualidad existen multitud de estudios que han evaluado los efectos de diferentes disruptores endocrinos en organismos acuáticos, incluyendo mamíferos. En la siguiente tabla se resumen algunos de los efectos observados para diferentes organismos, producidos por compuestos disruptores endocrinos y descritos por diversos autores [38–43].

Tabla 1.2. Efectos producidos por diferentes compuestos disruptores endocrinos en distintos organismos

<i>Invertebrados</i>		
<b>Disruptor endocrino</b>	<b>Especie afectada</b>	<b>Efecto</b>
Bisfenol-A	Mosquito	Problemas de desarrollo
Octilfenol	Anélidos	Metamorfosis prematura de las larvas
Diferentes pesticidas	Copépodos marinos	Anomalías en el desarrollo
<i>Peces, aves y reptiles</i>		
<b>Disruptor endocrino</b>	<b>Especie afectada</b>	<b>Efecto</b>
p-p'-DDE	Caimán	Desmasculinización
Perclorato	Carpita cabezona	Pérdida de pigmentación y menor masa corporal
Trenbolona		Disminución de la respuesta inmune y antiinflamatoria
Tributilestaño (TBT)	Falso halibut del Japón	Disminución de la actividad de los lisozimas y del número de linfocitos
Bisfenol-A	Trucha arcoíris	Incremento en el número de leucocitos. Cambios en las proteínas del sistema inmune
Nonilfenol	Pez cebra	Disminución en el número de leucocitos.
Dieldrin	Ánade real	Cambios en el comportamiento
Organofosfatos y carbamatos	Tordo sargento	Cambios de patrones de alimentación

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Tabla 1.2. (cont.) Efectos producidos por diferentes compuestos disruptores endocrinos en distintos organismos

<i>Mamíferos</i>		
Disruptor endocrino	Especie afectada	Efecto
PCBs	Oso polar	Masculinización
	Nutria europea	Disfunción reproductora
		Mayor incidencia de lesiones e infecciones
		Niveles menores de retinol hepático
	Foca común	Disminución de la fecundidad, problemas de implantación del embrión
Niveles menores de vitamina A y hormonas tiroideas		
Dioxinas	Visón, nutria	Problemas reproductivos
DDT	Foca gris	Osteoporosis
	Foca común	Lesiones craneales
Mercurio	Puma norteamericano	Trastorno del desarrollo de los órganos sexuales (criptorquidia)
	Foca anillada del Saimaa	Disminución de la población
Bisfenol-A	Topillo agreste	Incremento de los niveles de testosterona
Lindano y trifuralin	Oveja	Incremento de los niveles de insulina y estradiol

### 1.3. Las hormonas esteroideas

#### 1.3.1. Características generales y presencia en el cuerpo humano

Los esteroides son un grupo de compuestos químicos de gran interés por la gran variedad de productos que existen, la importancia biológica de la mayor parte de ellos, sus actividades fisiológicas y, por ende, sus aplicaciones farmacológicas. Este grupo de compuestos orgánicos está formado por el colesterol, del que derivan la mayoría de los

demás esteroides, otros esteroides, los ácidos biliares, las hormonas sexuales y las producidas en la corteza suprarrenal [44].

Estos dos últimos tipos de esteroides, las hormonas sexuales y las producidas en la corteza suprarrenal, se conocen como hormonas esteroideas y son los compuestos que se han estudiado en la presente Tesis Doctoral, ya que son dos de los grupos de mayor relevancia dentro de los disruptores endocrinos. Este grupo de moléculas biológicamente activas se sintetizan a partir del colesterol y tienen en común un núcleo fundamental formado por un fenantreno hidrogenado y un ciclopentano. En la Figura 1.4. se muestra esta formación de 17 átomos de carbono, la cual se conoce como ciclopentanoperhidrofenantreno, esterano o gonano, así como la numeración de los diferentes carbonos que forman la estructura y las letras con las que suelen designar a los anillos que la forman.

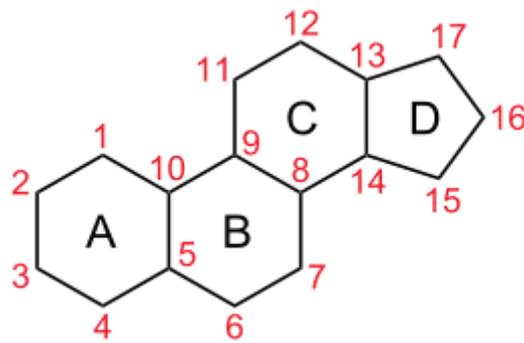


Figura 1.4. Molécula de ciclopentanoperhidrofenantreno

El núcleo o esqueleto de las hormonas esteroideas es bastante rígido, con la particularidad de que es prácticamente plano, y las diferencias entre esteroides estriban en la presencia de grupos funcionales tanto hidrófobos (cadenas alifáticas) como hidrófilos (carbonilos e hidroxilos). Las hormonas esteroideas suelen presentar

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grupos metilos en las posiciones 10 y 13, grupos carbonilo o hidroxilo en el C<sub>3</sub> o cadenas laterales de hidrocarburo en la posición 17. La presencia de unos u otros grupos funcionales determina el tipo de receptor celular al que pueden unirse las diferentes hormonas esteroideas, lo cual establece su clasificación en cinco grupos: estrógenos, andrógenos, progestágenos, glucocorticoides y mineralocorticoides [44,45]. Debido a la estabilidad del esqueleto esterano de las hormonas esteroideas, las células de los animales no son capaces de catabolizarlo, por lo que la conversión de hormonas activas se realiza principalmente alterando dichos grupos funcionales sustituyentes [46].

Las hormonas esteroideas de origen natural se sintetizan mayoritariamente en el tejido suprarrenal, ovarios, testículos y placenta. Cuando son excretadas, una vez llegan al medio ambiente, al ser moléculas biológicamente activas, pueden activar las células receptoras de los organismos presentes en los sistemas medioambientales, produciendo cambios en los sistemas endocrinos de éstos. Por esta razón son consideradas como un importante grupo de compuestos disruptores del sistema endocrino.

Además de la clasificación según la estructura de las diferentes hormonas esteroideas, se pueden diferenciar dos grandes grupos, dependiendo de dónde sean producidas. De esta manera, las hormonas sexuales y progestacionales son aquellas que han sido segregadas en los órganos sexuales, tanto femeninos como masculinos. A este grupo pertenecen los estrógenos, que son aquellas hormonas sexuales de carácter femenino, los andrógenos, de carácter masculino y los progestágenos, que son las hormonas ligadas a la gestación. El otro gran grupo los conforman las hormonas esteroideas producidas en las

glándulas suprarrenales, que a su vez se dividen en mineralocorticoides y glucocorticoides. Aparte de estas hormonas naturales, en ambos grupos encontramos hormonas sintéticas, diseñadas por el ser humano que han sido empleadas desde hace décadas para diversas funciones.

Las hormonas esteroideas suelen encontrarse en el plasma del torrente sanguíneo y pueden distribuirse en tres estados: libres, es decir, deconjugadas y, por tanto, en forma desligada; biodisponibles, lo que significa que están ligadas a proteínas transportadoras de baja afinidad; e inactivas, cuando se encuentran unidas a proteínas con una alta afinidad, como podrían ser la globulina o la  $\alpha$ -fetoproteína [47]. De estos tres estados, las hormonas libres y las biodisponibles son fisiológicamente activas, mientras que las inactivas, al estar ligadas a proteínas de alta afinidad, no producen efectos biológicos [48].

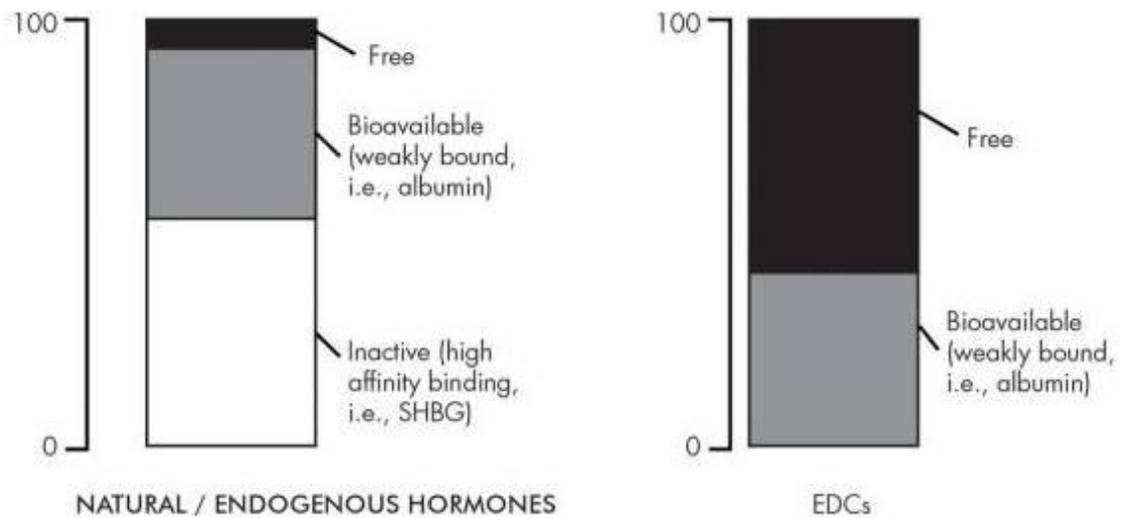


Figura 1.5. Distribución de las diferentes formas en que se presentan las hormonas y los compuestos disruptores endocrinos en sangre. Figura extraída de ref. [41].

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En el cuerpo humano, la mayoría de las hormonas se encuentran en estado inactivo o biodisponible, y sólo una pequeña parte se presenta en forma libre. Por el contrario, en el caso de los disruptores endocrinos, tal y como se puede comprobar en la Figura 1.5, la mayoría de ellos son fisiológicamente activos, al encontrarse en forma libre o biodisponible. La relación de los tres estados en los que se presentan las hormonas forma un sistema regulado (denominado *buffering*) que permite que las hormonas estén disponibles en el plasma sanguíneo y que las concentraciones en formas fisiológicamente activas no sean elevadas. En el caso de la presencia de compuestos disruptores endocrinos, el sistema deja de estar regulado, provocando que haya mayor cantidad de compuestos biológicamente activos, y, por tanto, cambiando el balance de las hormonas endógenas del organismo.

Las concentraciones a las que se encuentran las hormonas en el organismo son muy bajas, en el rango de micromolar a picomolar, y variables, presentando tanto ciclos o ritmos hormonales que dependen del momento del día, la época del año y el ciclo sexual, así como niveles más altos en determinados eventos fisiológicos determinados por condiciones externas al organismo [11]. Sin embargo, y aunque pudieran parecer arbitrarios, estos cambios están perfectamente regulados por el sistema endocrino. En la tabla 1.3. se resumen las concentraciones de algunas hormonas sexuales endógenas (estrógenos, andrógenos y progesterona) en hombres y mujeres en diferentes etapas de la vida [49–51].

Tabla 1.3. Concentraciones endógenas ( $\text{pg}\cdot\text{mL}^{-1}$ ) de algunas hormonas sexuales en hombres y mujeres en diferentes etapas de la vida.

		Mujeres			Hombres	
		Pre-pubertad	Adultas	Post-menopausia	Pre-pubertad	Adultos
Estradiol	Libre		0,5 - 9	0,05 - 0,2		
	Total	< 20	20 - 800	< 30		10 - 60
Estrona				14 - 34		36 - 38
Testosterona	Libre		9 - 150	1 - 4		300 - 250·10 <sup>3</sup>
	Total			110 - 330		
Androstenediona				310 - 1030		768 - 1128
DHEA				1160 - 4730		1191 - 3103
Progesterona		2,0 - 5,5	0,2 - 40	15 - 100	100 - 400	200 - 2000

### 1.3.2. Fuentes de emisión, transporte y presencia en el medio ambiente

Una de las características principales de las hormonas esteroideas es que se excretan continuamente a través de las heces y la orina de los organismos [52], lo cual genera un importante problema para los compartimentos ambientales receptores, pues, aunque las concentraciones son muy bajas, la entrada de este tipo de contaminantes es continua. Esto, ligado al hecho de que las hormonas pueden provocar disrupción en los sistemas endocrinos a concentraciones de  $\text{ng}\cdot\text{L}^{-1}$ , hace necesario el control de las fuentes de emisión, así como las vías de transporte de estos contaminantes a través del medio.

Teniendo en cuenta que las hormonas esteroideas se eliminan a través de las heces y la orina, la fuente principal de emisión de hormonas son las estaciones depuradoras de aguas residuales [53–56]. El problema en el caso de las hormonas esteroideas, al igual que con otros

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contaminantes emergentes, estriba en que los sistemas de depuración no están diseñados para eliminar estos contaminantes y que actualmente no existe una legislación que establezca límites de concentración para la mayoría de ellas en las aguas. De hecho, en la actual Directiva Marco del Agua de la Unión Europea no existen concentraciones límite de hormonas esteroideas en aguas, a excepción de tres estrógenos, el  $17\beta$ -estradiol, el  $17\alpha$ -etinilestradiol y la estrona [4,57].

Además de las EDARs, en la Figura 1.6 se muestran otras fuentes de emisión de hormonas esteroideas al medio acuático como los efluentes de zonas hospitalarias, que presentan elevadas concentraciones de hormonas debido a las diversas aplicaciones farmacológicas que éstas presentan, las aguas de desecho de ganadería y acuicultura, ya que, aunque las hormonas esteroideas están prohibidas para engordar al ganado [58], pueden utilizarse con fines veterinarios, las aguas de desechos de industrias farmacéuticas o vertidos incontrolados y pérdidas de los sistemas de saneamiento de aguas residuales urbanas [6].

Tanto las hormonas endógenas, como las sintéticas utilizadas como fármacos, se excretan a través de la orina como compuestos conjugados inactivos, los cuales, una vez degradados por las bacterias presentes en el medio, se convierten en sus formas libres o activas. Sin embargo, las bacterias presentes en las heces como la *Escherichia coli* pueden degradar grandes cantidades de la enzima  $\beta$ -glucuronidase, haciendo que las hormonas conjugadas como compuestos glucurónidos lleguen al medio como moléculas biológicamente activas [59].

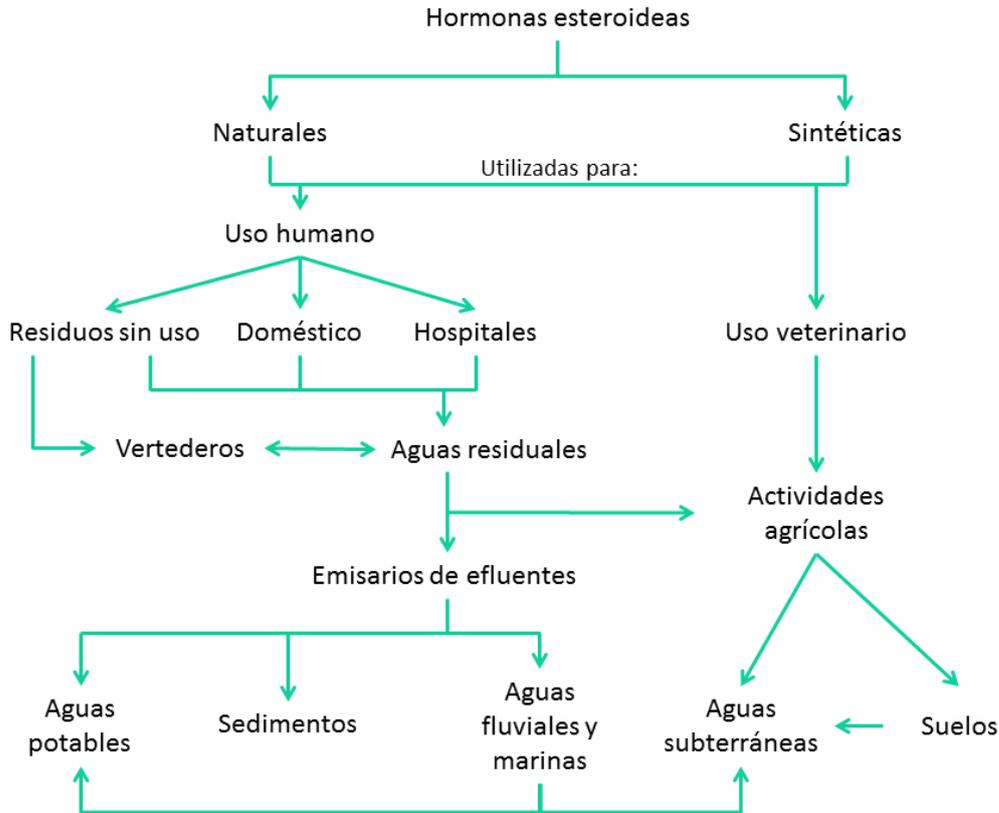


Figura 1.6. Diagrama de las diferentes vías de entrada de hormonas esteroideas al medio ambiente.

Una vez las hormonas esteroideas llegan al medio acuático pueden difundirse a través de éste mediante diferentes métodos de transporte [60]. El método principal de transferencia de la fase disuelta a la fase sólida es mediante adsorción sobre ésta. Para establecer la capacidad de transferencia (tanto por adsorción como por absorción) que se producirá en sedimentos se utiliza coeficiente de reparto sólido-agua ( $K_d$ ), el cual se define según la siguiente fórmula:

$$K_d = \frac{C_{sólido}}{C_{agua}} \quad (1)$$

Donde las concentraciones tanto en el sólido como en agua se definen como  $\text{mol}\cdot\text{kg}^{-1}$ , y  $\text{mol}\cdot\text{L}^{-1}$ . Este coeficiente de reparto se puede

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relacionar con el coeficiente de reparto sólido-agua normalizado para la cantidad de carbono orgánico de la muestra sólida ( $K_{OC}$ ) y el porcentaje de carbono orgánico de la muestra según la siguiente ecuación ( $f_{OC}$ ):

$$K_d = K_{OC} \cdot f_{OC} \quad (2)$$

Teniendo en cuenta que muchos estudios han relacionado los valores de  $K_{OC}$  con los valores del coeficiente de partición octanol-agua ( $K_{OW}$ ), se puede hacer una predicción de la adsorción de hormonas que se producirá en un sedimento o suelo determinado. Además del carácter hidrófobo de las hormonas, es necesario conocer su solubilidad en agua así como su presión de vapor para establecer la probabilidad de que dichos compuestos pasen de la fase disuelta o suspendida, a la fase sólida del medio receptor [61]. En la Tabla 1.4. se establecen los valores de diferentes parámetros físico-químicos para las hormonas estudiadas en la presente Tesis Doctoral, extraídos de las bases de datos HSDB (*Hazardous Substances Data Bank*) y Scifinder [62,63].

Tal y como se puede apreciar en la tabla, las bajas presiones de vapor que presentan este tipo de compuestos indican que es poco probable que se evaporen o volatilicen y pasen al medio aéreo, manteniéndose en las fases líquida o sólida, donde, además, teniendo en cuenta los valores relativamente bajos de solubilidad en agua, precipitarán hacia las fases sólidas. Por otra parte, teniendo en cuenta los coeficientes de reparto octanol-agua que presentan las sustancias estudiadas en la presente Tesis Doctoral, es lógico asumir que este tipo de disruptores endocrinos tenderán a pasar de la fase líquida a la fase sólida. Además, debido a este carácter lipófilo, tenderán a permanecer fuertemente adsorbidos en los tejidos grasos de los organismos presentes en el medio,

produciéndose fenómenos de bioacumulación en organismos acuáticos [64,65]

Tabla 1.4. Características fisicoquímicas de las hormonas esteroideas estudiadas en esta Tesis Doctoral

		Log K <sub>ow</sub>	Presión de vapor (mmHg)	Solubilidad en agua (mg·L <sup>-1</sup> )
<b>Estrógenos</b>	17β-estradiol	4,01	6,4·10 <sup>-9</sup>	3,0
	17α-etinilestradiol	3,67	1,9·10 <sup>-9</sup>	11,3
	Estriol	2,45	9,9·10 <sup>-12</sup>	27,3
	Estrona	3,13	2,5·10 <sup>-10</sup>	3,0
	Dietilestilbestrol	5,07	3,3·10 <sup>-7</sup>	12,0
<b>Andrógenos</b>	Testosterona	3,32	1,7·10 <sup>-8</sup>	23,4
	Nandrolona	2,62	3,5·10 <sup>-8</sup>	24,0
	Boldenona	3,09	2,0·10 <sup>-9</sup>	20,0
<b>Progestágenos</b>	Progesterona	3,87	3,54·10 <sup>-4</sup>	8,8
	Noretisterona	2,97	3,1·10 <sup>-7</sup>	7,0
	Levonorgestrel	3,48	3,9·10 <sup>-10</sup>	2,1
	Acetato de megestrol	3,75	2,1·10 <sup>-10</sup>	6,5
<b>Glucocorticoides</b>	Cortisona	1,43	3,0·10 <sup>-15</sup>	140,0
	Prednisona	1,46	3,8·10 <sup>-13</sup>	77,5
	Prednisolona	1,62	1,2·10 <sup>-13</sup>	223,0

Teniendo todas estas propiedades fisicoquímicas en cuenta, se podría afirmar que es bastante probable que las hormonas esteroideas pasen de la fase acuosa en la que suelen llegar al medio, a la fase sólida. Sin embargo, la presencia de hormonas en la fase líquida está bien

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documentada en la bibliografía, ya que existen diversos mecanismos que pueden hacer que la difusión de estos contaminantes hacia las fases sólidas no se produzca, permaneciendo éstos en la masa de agua. Estos mecanismos son la formación de coloides que aumentan la estabilidad de estos compuestos en la fase disuelta, la formación de metabolitos o compuestos intermedios más solubles, como los conjugados sulfatos o glucurónidos, o el incremento de solubilidad en medios con pH alto debido a que la mayoría de las hormonas esteroideas presentan valores de  $pK_a$  superiores a 10. Aparte de llegar a las matrices sólidas desde las líquidas, las hormonas esteroideas pueden adsorberse sobre suelos agrícolas en los que se utilizan residuos ganaderos como abono, o por difusión desde masas de agua cercanas [66,67]. Además, diversos estudios han determinado la correlación directa entre la adsorción de hormonas sobre suelos y el contenido de carbono orgánico que éstos presentan [68,69], aunque la presencia de carbono orgánico no es un requisito fundamental para que se produzca adsorción, ya que las propiedades fisicoquímicas de las hormonas esteroideas las hacen más propensas a depositarse sobre la fase sólida [69].

Según estos mecanismos de transporte, y, teniendo en cuenta la entrada continua de hormonas esteroideas al medio, es una realidad que en la actualidad se encuentren concentraciones apreciables de este tipo de disruptores endocrinos en diversos compartimentos ambientales. Debido a las características propias de cada tipo de matriz, el estudio y seguimiento de hormonas en matrices líquidas está bastante más desarrollado que el estudio en matrices sólidas. En la tabla 1.5. se recogen los resultados obtenidos por algunos estudios en los que se han analizado hormonas esteroideas en diferentes matrices. De manera general, las

mayores concentraciones de hormonas detectadas se dan en influentes de estaciones depuradoras de aguas residuales, siendo las concentraciones algo más bajas a la salida de la EDAR, estando en ambos casos en el rango de  $\text{ng}\cdot\text{L}^{-1}$  a  $\mu\text{g}\cdot\text{L}^{-1}$ . En las aguas medioambientales, ya sean fluviales o marinas, las concentraciones detectadas por los diferentes estudios suelen ser más bajas, en el rango de los  $\text{ng}\cdot\text{L}^{-1}$ , debido principalmente al factor de dilución que sufren los efluentes de las EDAR cuando son vertidos al medio.

Tabla 1.5. Concentraciones de hormonas esteroideas detectadas en diversas matrices medioambientales

Localización de las muestras	Matriz analizada	Hormona esteroidea estudiada	Concentraciones detectadas	Ref. y año
<b>Muestras líquidas</b>				
China	Agua de lago	17 $\beta$ -estradiol	2,52 – 21,8 $\text{ng}\cdot\text{L}^{-1}$	[70]
		17 $\alpha$ -etinilestradiol	4,25 – 12,85 $\text{ng}\cdot\text{L}^{-1}$	(2017)
Estados Unidos	Agua subterránea	Estrona	40 - 390 $\text{ng}\cdot\text{L}^{-1}$	[71]
		Testosterona	30 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	(2011)
	Agua de río	Estrona	0,90 – 2,90 $\text{ng}\cdot\text{L}^{-1}$	[72]
	Agua salada	Estrona	0,66 – 5,2 $\text{ng}\cdot\text{L}^{-1}$	
		17 $\beta$ -estradiol	nd <sup>b</sup> – 1,8 $\text{ng}\cdot\text{L}^{-1}$	(2010)
Francia y Bélgica	Agua de río	Estrona	34,2 – 116,2 $\text{ng}\cdot\text{L}^{-1}$	[73]
		Testosterona	5,8 – 6,5 $\text{ng}\cdot\text{L}^{-1}$	
		Estriol	21,0 – 77,7 $\text{ng}\cdot\text{L}^{-1}$	
		Progesterona	5,7 – 10,9 $\text{ng}\cdot\text{L}^{-1}$	
Francia	Agua superficial	Testosterona	2,2 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	[74]
		Noretisterona	2,0 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	
		Levonorgestrel	3,6 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	
		Progesterona	1,6 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	
	Agua subterránea	Testosterona	1,4 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	
		Noretisterona	1,9 $\text{ng}\cdot\text{L}^{-1}$ <sup>a</sup>	

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Tabla 1.5. (cont.) Concentraciones de hormonas esteroideas detectadas en diversas matrices medioambientales

Localización de las muestras	Matriz analizada	Hormona esteroidea estudiada	Concentraciones detectadas	Ref. y año
<b>Muestras sólidas y biológicas</b>				
<b>China</b>	Sedimentos de lago	17 $\beta$ -estradiol	0,52 – 1,21 ng·g <sup>-1</sup>	[70] (2017)
		17 $\alpha$ -etinilestradiol y dietilestilbestrol	0,61 – 1,48 ng·g <sup>-1</sup>	
	Peces	17 $\beta$ -estradiol	0,85 ng·g <sup>-1 a</sup>	
		17 $\alpha$ -etinilestradiol y dietilestilbestrol	0,11 – 1,18 ng·g <sup>-1</sup>	
<b>Serbia</b>	Sedimentos de río	Mestranol	10 ng·g <sup>-1 a</sup>	[75] (2011)
<b>Suiza</b>	Leche de vaca	17 $\beta$ -estradiol	5 – 58 ng·kg <sup>-1</sup>	[76] (2016)
		Estrona	5 -2181 ng·kg <sup>-1</sup>	
		Testosterona	30 – 38 ng·kg <sup>-1</sup>	
		Progesterona	86 – 42946 ng·kg <sup>-1</sup>	
		Cortisona	19 – 881 ng·kg <sup>-1</sup>	

<sup>a</sup> Valores medios

<sup>b</sup> no detectado

Otros estudios han determinado también la presencia de hormonas en aguas subterráneas y superficiales, las cuales no suelen ser medios receptores de hormonas esteroideas, pero que pueden verse contaminados indirectamente, en concentraciones comprendidas entre pg·L<sup>-1</sup> y ng·L<sup>-1</sup>.

En lo que respecta a muestras sólidas, aunque la bibliografía es más limitada, se observan resultados similares a las muestras líquidas, ya que las concentraciones más altas se detectan en lodos de EDAR (en el rango de  $\mu\text{g}\cdot\text{g}^{-1}$ ), mientras que las concentraciones detectadas en sedimentos de

sistemas acuáticos suelen ser moderadamente más bajas, encontrándose en el rango de  $\text{ng}\cdot\text{g}^{-1}$ . Finalmente, en organismos acuáticos, las concentraciones de hormonas, tanto naturales como sintéticas se encuentran a niveles traza y ultratrazas y, aunque no existen estudios de biomagnificación en la cadena trófica, sí se ha observado que las concentraciones detectadas en predadores situados en la parte alta de la cadena son superiores a las especies situadas en niveles tróficos inferiores.

### 1.3.3. Hormonas estudiadas

Como se ha expuesto en el apartado 1.3.1., existen diversas familias de hormonas esteroideas, dependiendo de su estructura y de los efectos que tienen sobre el organismo. Por esta razón es necesario conocer las características de cada una de estas familias, así como las hormonas que se han estudiado dentro de cada una de ellas. En la figura 1.7 se muestra un diagrama con los mecanismos de formación de algunas de las hormonas más importantes en el cuerpo humano.

#### 1.3.3.1. Estrógenos

Los estrógenos son hormonas sexuales esteroideas de tipo femenino, aunque se presentan en organismos de ambos sexos, producidas principalmente por los ovarios y en menor medida por las glándulas adrenales y otros sistemas biológicos. Debido al carácter lipófilo de las hormonas, pueden atravesar la pared de las células para interaccionar con los receptores estrogénicos (ER) que se encuentran en el núcleo celular y provocar así la formación de proteínas que serán las responsables de cambios en los órganos diana [77,78].

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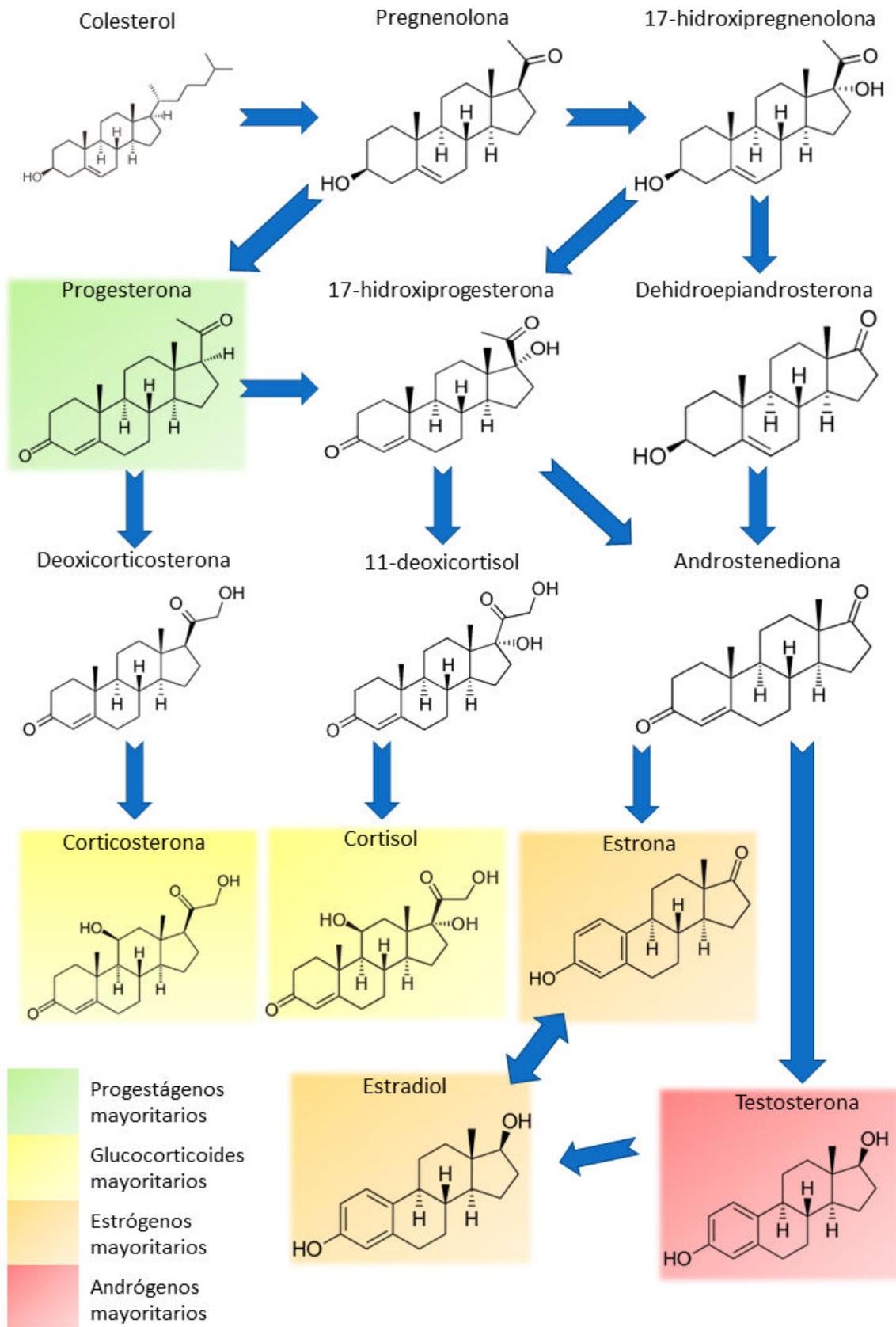


Figura 1.7. Esteroidogénesis de algunas de las hormonas más importantes en el cuerpo humano

En lo que respecta a su estructura, los estrógenos poseen un anillo aromático en su molécula (anillo A) y un grupo hidroxilo unido al C<sub>3</sub>, lo cual es característico a todos los estrógenos ya que es el lugar de fijación de la molécula a los ER de las células diana. Además, todos los estrógenos presentan un grupo metilo en el C<sub>13</sub> y, al contrario que otras hormonas esteroideas, carecen de grupo metilo en el C<sub>10</sub>, por lo que su estructura básica está formada por 18 átomos de carbono.

Debido al efecto de los estrógenos sobre el organismo, su uso farmacológico está ampliamente extendido, ya que tienen efectos sobre los órganos sexuales femeninos y son los responsables del desarrollo de los caracteres femeninos. [78,79]. Además, tienen acciones metabólicas, controlando elementos fundamentales como el calcio y el nitrógeno o afectando a los lípidos sanguíneos. Por estas razones pueden ser utilizados no sólo en terapias de reemplazo hormonal sino como fármacos en el tratamiento de cáncer de mama o metastásico postmenopáusico, de la osteoporosis, del acné o como inhibidores de procesos de arteriosclerosis [80,81]. Por su parte, los estrógenos sintéticos tienen como aplicación fundamental el control de la natalidad [82].

Una vez administrados, los estrógenos presentan una absorción irregular ya que sufren inactivación por parte de las bacterias gastrointestinales y son metabolizados principalmente en el hígado, donde se conjugan con el ácido glucurónico y sulfúrico, formándose conjugados biológicamente inactivos.

Teniendo en cuenta lo anterior, se establecen como principales fuentes de emisión de estos contaminantes al medio las estaciones

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depuradoras de aguas residuales y, en menor medida, los residuos ganaderos como los lixiviados del estiércol y los vertidos incontrolados. Pese a ser excretados como subproductos conjugados y menos activos que los originales, los estrógenos pueden reactivarse en sus formas libres, ya que las bacterias utilizadas en los métodos de depuración degradan en primera instancia los grupos funcionales glucurónidos y sulfatos. De manera general, se ha determinado que las EDARs son capaces de degradar gran parte de las hormonas presentes en el agua, pero no totalmente [83–85], necesitándose tecnologías avanzadas de oxidación o adsorción para la completa eliminación de éstos [86,87] y produciéndose además subproductos clorados, tras el tratamiento con cloro, los cuales también presentan actividad estrogénica, aunque menor que la de los productos principales [88]. Diversos estudios han determinado que la degradación producida por los tratamientos biológicos de las EDARs depende de las condiciones en las que éstos se realicen, comprobándose que se produce una degradación de las moléculas muchos más rápida de tratamientos aerobios que en tratamientos anaerobios [89].

Al contrario que ocurre con el resto de hormonas esteroideas, en la actualidad existe una legislación limitada acerca de la presencia de estrógenos en el medio. En Europa existe una lista de vigilancia (*Watch-List*) asociada a la Directiva Marco del Agua cuya función es recabar información para hacer que estos contaminantes emergentes pasen a ser considerados prioritarios dentro de la legislación comunitaria [4]. De todos los estrógenos existentes, sólo los estrógenos naturales  $17\beta$ -estradiol y estrona y el estrógeno sintético  $17\alpha$ -etinilestradiol se encuentran recogidos en esta lista de vigilancia. La inclusión de estos tres estrógenos está ligada al potente efecto estrogénico que poseen y servirá como punto

de partida para la futura legislación del resto de hormonas esteroideas en matrices ambientales.

El control de los estrógenos presentes en el medio se hace vital ya que son compuestos muy activos y que pueden producir cambios en los organismos expuestos, incluso a concentraciones traza (en el rango de  $\text{pg}\cdot\text{L}^{-1}$  a  $\text{ng}\cdot\text{L}^{-1}$ ). Ya desde los años 90 se conocen tanto el efecto estrogénico de los efluentes de las EDARs, así como los efectos sobre los organismos presentes en las aguas receptoras. Así, Purdom y colaboradores [90] determinaron el incremento de los niveles de vitelogenina en peces expuestos a efluentes de EDAR, lo cual es indicativo de estimulación estrogénica del hígado. Aunque en este primer estudio no se determinó la sustancia que provocaba esta actividad estrogénica, se atribuyó entre otras al estrógeno sintético  $17\alpha$ -etinilestradiol.

Estudios más recientes han determinado que los estrógenos, tanto naturales como sintéticos son los causantes de cambios relacionados con la sexualidad de organismos acuáticos como hermafroditismo o desarrollo de poblaciones unisexuales [91,92], así como efectos en otros sistemas biológicos de estos organismos, como por ejemplo cambios en el comportamiento, en la biomasa o en los tejidos duros de éstos [93–95].

En la presente Tesis Doctoral se han estudiado cinco estrógenos, de los cuales, tres de ellos son estrógenos endógenos, el  $17\beta$ -estradiol, la estrona y el estriol, los cuales presentan relación de interconversión entre ellos; uno es un estrógeno sintético, el  $17\alpha$ -etinilestradiol y el quinto es un estrógeno no esteroideo, el dietilestilbestrol. En la Figura 1.8 se muestran las diferentes estructuras moleculares de los estrógenos estudiados, en las

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que se puede comprobar que todos ellos son estrógenos esteroideos, a excepción del dietilestilbestrol.

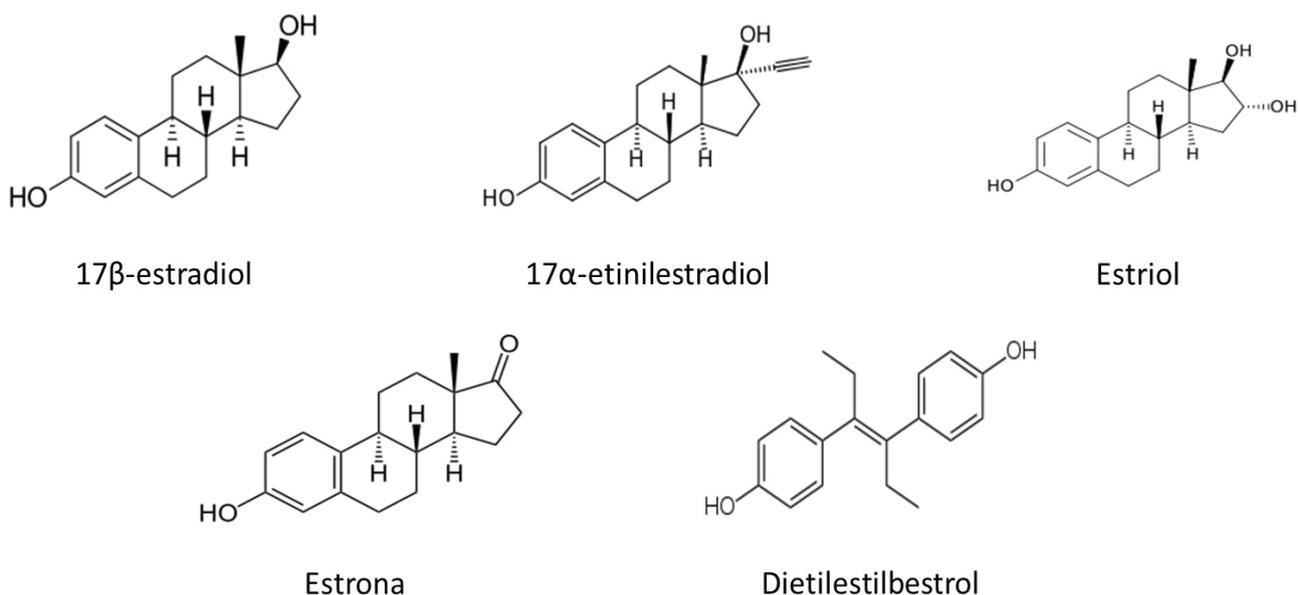


Figura 1.8. Estructuras moleculares de los estrógenos estudiados en la presente Tesis Doctoral

El 17β-estradiol se sintetiza principalmente en los ovarios y es una de las hormonas del desarrollo sexual de la mujer, siendo el estrógeno predominante en ésta durante la etapa reproductiva [96]. Además de sintetizarse en los ovarios, también se produce en el tejido adiposo, en las paredes arteriales y en el cerebro. En los hombres también está presente debido a que es un compuesto intermedio en la metabolización de la testosterona. Estructuralmente presenta dos grupos hidroxilo en las posiciones C<sub>3</sub> y C<sub>17</sub>, razón por la cual se suele abreviar como E2. Es el estrógeno natural más potente del cuerpo humano, y durante el proceso de desactivación que tiene lugar en el cuerpo humano se convierte en estrona y estriol, los cuales presentan menor actividad estrogénica.

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La estrona, también denominada foliculina, es el siguiente estrógeno del cuerpo humano en cuanto a capacidad estrogénica, la cual es unas 10 veces inferior a la del  $17\beta$ -estradiol. Es el estrógeno menos abundante en el cuerpo de la mujer en edad reproductiva, donde se suele encontrar en forma de sulfato de estrona ya que esta forma es la más adecuada para su transformación en  $17\beta$ -estradiol. Sin embargo, después de la menopausia pasa a ser el estrógeno mayoritario, llegando a ser su concentración entre el doble y cuatro veces más que la del  $17\beta$ -estradiol [97]. Estructuralmente sólo posee un grupo hidroxilo unido al  $C_3$ , por lo que suele abreviarse como E1.

En lo que respecta al tercero de los estrógenos naturales, el estriol, es el que menos potencia estrogénica presenta, siendo ésta unas 80 veces inferior a la del  $17\beta$ -estradiol. Se considera un estrógeno de acción breve, ya que su tiempo de retención en el núcleo de las células es corto [98] y su presencia en el torrente sanguíneo deriva de la metabolización tanto del  $17\beta$ -estradiol como de la estrona, siendo el estrógeno predominante en el cuerpo de la mujer durante el embarazo, (las concentraciones de estriol en orina pueden llegar a ser 1000 veces superiores a las concentraciones en mujeres no embarazadas). Como fármaco se utiliza ampliamente para contrarrestar el déficit estrogénico durante la menopausia, debido a la disminución de la producción de  $17\beta$ -estradiol y se ha comprobado su efectividad en el tratamiento de síntomas genitourinarios. Estructuralmente presenta tres grupos hidroxilo unidos a los carbonos  $C_3$ ,  $C_{16}$  y  $C_{17}$ , razón por la cual se suele abreviar como E3.

El  $17\alpha$ -etinilestradiol es un estrógeno sintético ampliamente utilizado en la actualidad [99]. Estructuralmente su molécula es

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exactamente igual a la del  $17\beta$ -estradiol, con la única diferencia de la adición de un grupo etinil en el  $C_{17}$ . Fue sintetizado en los años 30 por Hans Inhofenn y Walter Hohlweg, quienes comprobaron que la adición del grupo etinil producía que la desactivación de la molécula producida por las enzimas del hígado fuera más lenta, por lo que su actividad estrogénica era más duradera que la de las hormonas naturales [82]. Al igual que en el caso del  $17\beta$ -estradiol, una vez metabolizado, forma complejos glucurónidos y sulfatos que se excretan en la orina y las heces. En la actualidad sigue usándose no sólo en terapias de remplazo hormonal o en el tratamiento de hipogonadismo femenino, sino como anticonceptivo, normalmente en presencia de un progestágeno [100].

Finalmente, el quinto estrógeno estudiado en la presente Tesis Doctoral es el dietilestilbestrol. Este compuesto, pese a ser considerado un estrógeno, no presenta la estructura basada en los cuatro anillos que forman el esqueleto esteroideo. Su estructura deriva del compuesto natural anetol, que presenta una leve actividad estrogénica y se encuentra en el regaliz o el anís. Fue sintetizado en 1938 por investigadores ingleses quienes se percataron de que el anillo fenantreno no era necesario para desencadenar la actividad estrogénica, sino que era suficiente con el anillo bencénico unido a un grupo hidroxilo. De esta manera comenzaron a estudiar compuestos formados por dos anillos bencénicos unidos por una cadena carbonada, descubriendo que el dietilestilbestrol poseía una elevada capacidad estrogénica [82] equivalente a la del  $17\beta$ -estradiol o incluso superior a la de éste en el caso de administración por vía oral. Sin embargo, el dietilestilbestrol está contraindicado en mujeres embarazadas pues se demostró que podía ser el causante de malformaciones en las hijas de las mujeres que habían tomado este medicamento durante el

embarazo [101,102]. Otros usos como fármaco hasta finales de los años 90 fueron en el tratamiento de cáncer de mama o de próstata [103].

### **1.3.3.2. Andrógenos**

Los andrógenos constituyen el grupo de hormonas esteroideas que engloba a las hormonas sexuales de carácter masculino, las cuales son mayoritariamente segregados por los testículos, a excepción de una pequeña porción, en torno al 10%, que se segrega en las glándulas suprarrenales [104]. En el caso de la mujer, también se segrega una pequeña concentración de andrógenos, principalmente androstenediona, testosterona y dehidroepiandrosterona (DHEA), en los ovarios [105].

Los andrógenos se sintetizan a partir del colesterol y la pregnenolona, siendo sus intermediarios metabólicos la DHEA y la androstendiona. Al igual que ocurre con los estrógenos, los andrógenos tienen como esqueleto la molécula de ciclopentanoperhidrofenantreno, de 17 átomos de carbono presentando además dos grupos metilos en las posiciones C<sub>10</sub> y C<sub>13</sub>, por lo que la estructura básica de los andrógenos consta de 19 átomos de carbono. Además, pueden presentar una insaturación entre los carbonos C<sub>4</sub> y C<sub>5</sub>, un grupo hidroxilo y cetónicos en el C<sub>17</sub> y en el C<sub>3</sub>.

Los andrógenos son los responsables del desarrollo del aparato sexual y caracteres masculinos [104], lo cual se produce al unirse a los receptores androgénicos que se encuentran en el núcleo de las células diana [105,106]. Algunas acciones metabólicas producidas por los andrógenos son aumento de la síntesis de proteínas, cambios en el metabolismo de las grasas y los carbohidratos [107], o la formación de

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glóbulos rojos (eritropoyesis) mediante incremento en la producción renal. Además, poseen también actividad anabólica, que tiene como efecto principal el incremento de la masa muscular y estimulación de la síntesis de proteínas [104,108,109].

Debido a los efectos que producen en el organismo, los andrógenos tienen una gran aplicación en el campo terapéutico, en el tratamiento de enfermedades tan dispares como hipogonadismo, cáncer o anemia [110,111]. Además, debido a su carácter anabólico pueden ser utilizados para fomentar la ganancia de peso [112] y también se pueden utilizar para fomentar el desarrollo de la masa y rendimiento muscular en deportistas, razón por la cual muchos de ellos son considerados como sustancias dopantes [113,114]. Aunque la utilización de sustancias dopantes se remonta a la antigüedad, este aspecto se ha convertido en un verdadero problema en la actualidad, por lo que existe una amplia regulación acerca del uso de anabolizantes en deportistas. Tanto la Agencia Mundial Antidopaje (WADA, por sus siglas en inglés) como el Consejo Superior de Deportes en España, han prohibido en cualquier situación y deporte el uso de compuestos anabólicos tanto endógenos, como la testosterona o la nandrolona, como exógenos, como la oxabolona o el estanozolol, así como otros agentes anabólicos como los moduladores de los receptores androgénicos [115–117].

En el cuerpo humano, un pequeño porcentaje de andrógenos como la testosterona se encuentra en estado libre, mientras que más del 95% se encuentran ligados a proteínas y por lo tanto inactivos [108] y el metabolito principal de la testosterona que se excreta en la orina es la androsterona en forma de conjugados glucurónidos y sulfatos [104]. La

testosterona presenta una metabolización muy rápida en el cuerpo humano, mientras que otros andrógenos como la metiltestosterona se metabolizan más lentamente, pudiendo producir, por tanto, efectos androgénicos durante más tiempo.

En la Tabla 1.6 se muestran los valores medios de excreción de algunos andrógenos endógenos [118], por lo que es lógico determinar que la fuente principal de andrógenos, tanto endógenos como exógenos al medio ambiente serán las estaciones depuradoras de aguas residuales.

Tabla 1.6. Ratios de excreción de algunos andrógenos endógenos en hombres y mujeres

Andrógeno	Ratio de excreción ( $\mu\text{g}\cdot\text{d}^{-1}$ ) en hombres	Ratio de excreción ( $\mu\text{g}\cdot\text{d}^{-1}$ ) en mujeres
Testosterona	37,2 – 117,7	6,1 – 7,7
Dehidrotestosterona	14,1	- <sup>a</sup>
Epitestosterona	38,3 – 166,4	6,5 – 19,6
Androsterona	1864 – 3482	955,4 – 2860
4-androstenediol	302 – 650	203,3

<sup>a</sup> Sin datos

Pese a no estar tan estudiados como las hormonas femeninas, las concentraciones de andrógenos en el agua residual suelen ser mucho más altas que las de estrógenos. De hecho, Chang y colaboradores determinaron que la concentración de andrógenos constituía más del 95% de la concentración total de hormonas en el influente de siete estaciones depuradoras situadas en China [119]. Este fenómeno ha sido corroborado por otros estudios en los que también se ha comprobado que la degradación de andrógenos producida por los tratamientos de depuración de las EDARs es muy alta, con eficiencias superiores al 95% para la

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mayoría de ellos [64,120]. No obstante, pese a los altos ratios de eliminación de andrógenos en las aguas residuales, los residuos que pueden quedar en éstas pueden afectar a los ecosistemas acuáticos donde se vierten las aguas regeneradas. Los efectos sobre la fauna producidos por los andrógenos no están tan ampliamente analizados, pero algunos estudios han observado masculinización y disminución de la fertilidad de hembras de peces expuestos a andrógenos como la trenbolona [121,122]. Otros andrógenos como la 17 $\alpha$ -metiltestosterona son capaces de alterar otros sistemas biológicos de los organismos marinos aparte del sistema reproductor, produciendo cambios morfológicos como malformaciones de cabeza y cola de peces como el pez cebra o disminución de la capacidad enzimática antioxidativa de otros peces como la tilapia [123,124].

En la presente Tesis Doctoral se han estudiado tres andrógenos, dos de ellos naturales, la testosterona y la nandrolona y uno sintético, la boldenona cuyas estructuras moleculares se muestran en la Figura 1.9.

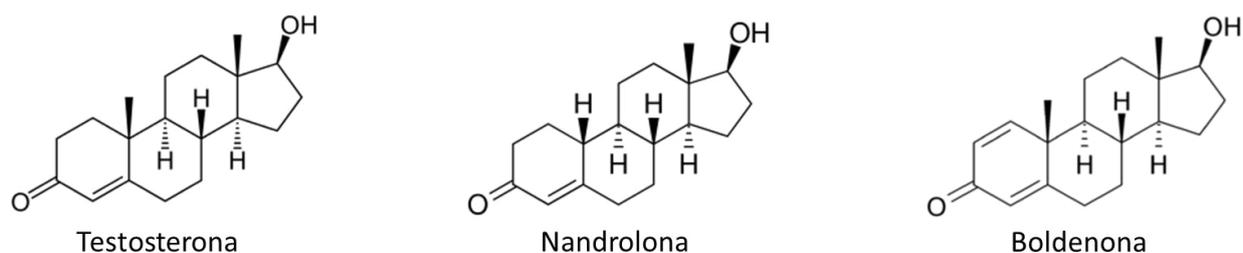


Figura 1.9. Estructuras moleculares de los andrógenos estudiados en la presente Tesis Doctoral

La testosterona es el principal andrógeno natural, el cual se produce principalmente en los testículos, concretamente en las células de Leydig y, en menor cantidad, en los ovarios y la corteza suprarrenal, donde se

encuentra bajo control de la corticotropina [125]. La testosterona presenta efectos anabólicos y androgénicos, los cuales no pueden ser separados, considerándose que la ratio entre estos efectos es 1:1. Además, es considerada como una prohormona, ya que como tal no es activa en el cuerpo humano, pero si lo es su metabolito activo, el 5 $\alpha$ -dihidrotestosterona (DHT), el cual induce las acciones androgénicas anteriormente descritas [104]. La testosterona se ve afectada por una rápida metabolización en el cuerpo, por lo que se han desarrollado multitud de fármacos basados en su estructura, y, por tanto, con propiedades similares, pero que sean más estables, pudiendo producir efectos en el organismo durante un tiempo más prolongado. Algunos ejemplo de este tipo de moléculas son el estanozolol o la propia metiltestosterona [126,127], en los que se ha producido la alquilación del grupo hidroxilo del C<sub>17</sub>, o compuestos en los que se esterifica ese mismo grupo hidroxilo, lo cual ocurre en los derivados de la nandrolona, la trembolona o la boldenona, entre otros [127].

El segundo de los andrógenos estudiados ha sido la nandrolona, la cual fue sintetizada en los años 50. Estructuralmente, la nandrolona es exactamente igual a la testosterona, con la única diferencia de que no posee un grupo metilo unido al C<sub>10</sub>, es decir, no tiene el C<sub>19</sub>, razón por la cual también se suele denominar como nortestosterona o 19-nortestosterona [128]. La metabolización de la nandrolona es muy similar a la de la testosterona, sufriendo primero la oxidación del grupo hidroxilo del C<sub>17</sub>, seguida de la reducción del anillo A, obteniéndose los isómeros norandrosterona y noreticolanolona, los cuales son utilizados para la determinación de dopaje por nandrolona [128] y que suelen excretarse como conjugados glucurónidos. La utilización de nandrolona como agente

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dopante es muy popular debido a que la relación entre efectos anabolizantes y androgénicos es muy alta, siendo detectada por la WADA como la segunda sustancia anabólica dopante más utilizada [126]. La nandrolona puede ser utilizada para el tratamiento de trastornos como la anemia y la pérdida de tono muscular producida por diversos trastornos [129,130], así como en tratamiento de hipogonadismo o alopecia androgénica [131].

Finalmente, el tercero de los andrógenos estudiados ha sido la boldenona. Este andrógeno sintético posee una estructura análoga a la de la testosterona, presentando además una insaturación entre los carbonos C<sub>1</sub> y C<sub>2</sub> y una ratio de efectos anabólicos-androgénicos muy alta. En su uso como fármaco, no se administra boldenona libre, la cual posee una vida media de unos 14 días, sino un compuesto derivado de ésta, el éster undecilenato de boldenona, ya que la adición de la cadena carbonada permite que la acción del andrógeno dure más, excretándose, una vez metabolizado, como un conjugado 17β-sulfato [132]. El uso del undecilenato de boldenona es eminentemente veterinario, aunque también se ha usado para el desarrollo muscular de culturistas [133]. Pese a estos usos, diversos estudios en los últimos años han determinado que la boldenona es probablemente un compuesto carcinógeno, presentando un índice carcinógeno superior a otros andrógenos anabolizantes como la nandrolona, el estanozolol o la testosterona [134].

### **1.3.3.3. Progesterona y progestágenos sintéticos**

Los progestágenos son hormonas femeninas que participan en muchos otros procesos metabólicos, especialmente la gestación, razón

por la cual también son denominados *gestágenos*. La progesterona es el principal progestágeno endógeno y es vital no sólo en procesos de embriogénesis o embarazo, sino también en fases de preparación de la mujer como el ciclo menstrual y la menstruación [78]. La progesterona se produce principalmente en la segunda mitad del ciclo menstrual y en el caso de los hombres, una pequeña parte también es sintetizada por los testículos. Por ello, pese a ser un tipo de hormona ligada al embarazo en la mujer, se considera como hormona sexual [135]. Los compuestos sintéticos con acción similar a la de la progesterona se denominan progestinas y se desarrollaron para solucionar problemas asociados a la progesterona cuando se utilizaba como fármaco [136]. Debido a la similitud de denominación, organismos como la *North American Menopause Society* (NAMS), recomiendan el uso del término *progestágeno* para todos aquellos compuestos tanto naturales como sintéticos que presentan actividad progestacional, mientras que el término *progestina* se reserva para los progestágenos de origen sintético [137].

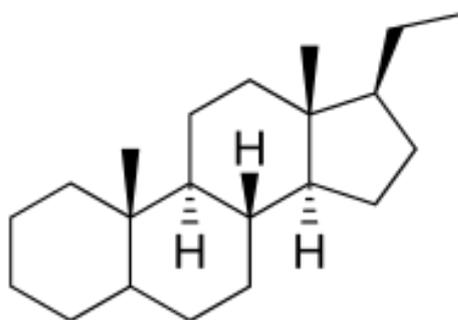


Figura 1.10. Estructura molecular común para los progestágenos denominada *pregnano*.

La progesterona presenta una estructura básica de 21 átomos de carbono denominado pregnano (Figura 1.10). Esta estructura básica

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comprende los 17 átomos de carbono del ciclopentanoperhidrofenantreno, así como los correspondientes a dos grupos metilos, los cuales se unen a los carbonos C<sub>10</sub> y C<sub>13</sub> y a un grupo acetilo unido al C<sub>17</sub>. Este grupo acetilo es la única diferencia entre la molécula de la progesterona y la de algunos andrógenos como la testosterona ya que, al igual que ellos, ésta presenta un grupo cetónico en el C<sub>3</sub> y una insaturación entre los carbonos C<sub>4</sub> y C<sub>5</sub> del anillo A. En el caso de las progestinas, la estructura de éstas puede estar basada en la molécula de la progesterona o bien en la de la testosterona, debido a las similitudes que hay entre ambas. Así se podría hacer una primera clasificación entre progestágenos naturales (progesterona) y sintéticos, los cuales a su vez podrían dividirse en progestinas basadas en la estructura de la progesterona y basadas en la estructura de la testosterona [138,139]. A su vez, tal y como muestra la Figura 1.11, estos dos grandes grupos pueden dividirse en subgrupos según las características estructurales de cada uno de ellos:



Figura 1.11. Clasificación de los progestágenos según su estructura

Los progestágenos, tanto naturales como sintéticos se unen a los receptores específicos situados en el núcleo de las células diana [137,140], sin embargo, debido a las similitudes estructurales de andrógenos y glucocorticoides con los progestágenos, estos últimos pueden también producir efectos androgénicos [141] o actuar como glucocorticoides o anti-mineralocorticoides débiles [142].

La progesterona, durante la segunda mitad del ciclo menstrual, se segrega en mayor cantidad para favorecer el desarrollo del endometrio y así prepararlo para una posible implantación del cigoto. Si la fecundación tiene lugar, la progesterona producida provocará diversos cambios como evitar la ovulación y disminuir las contracciones del útero para mantener el embarazo [136]. Además, inhibe la actividad estrogénica en la mujer y favorece la metabolización de estrógenos libres para que se conviertan en metabolitos biológicamente inactivos [140]. En lo que respecta a otros sistemas biológicos, la progesterona tiene una actividad termogénica [78], y, debido a su semejanza estructural a los andrógenos, posee una cierta actividad anabólica y androgénica. Además eleva los niveles basales de insulina y la respuesta de ésta a la glucosa y presenta actividad sobre el sistema nervioso central [136,137].

Los variados efectos sobre el organismo hacen que los progestágenos, sobre todo los sintetizados artificialmente, sean utilizados para el tratamiento de múltiples trastornos orgánicos. Las aplicaciones terapéuticas principales de los progestágenos son el tratamiento de déficit hormonal y como anticonceptivos [78]. En el caso de la progesterona, su uso como anticonceptivo es limitado puesto que la metabolización de ésta es muy rápida [143], mientras que las progestinas se han diseñado con

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estructuras capaces de impedir esta rápida metabolización, por lo que su uso como anticonceptivo está muy desarrollado. Algunos ejemplos de progestinas utilizadas como anticonceptivo son la noretisterona, el levonorgestrel o el desogestrel, los cuales derivan de la molécula de la 19-nortestosterona [138]. En lo que respecta a otros usos terapéuticos, la progesterona y los progestágenos sintéticos se han utilizado desde hace décadas para aliviar problemas ligados a la menstruación [78,136,140], en terapias de remplazo hormonal, o para prevenir hiperplasia y cáncer de endometrio los cuales se ven favorecidos por la presencia de estrógenos tras la menopausia [142].

Al contrario que ocurre con estrógenos y andrógenos, el metabolismo de los progestágenos está poco estudiado, aunque sí se sabe que, en el caso de la progesterona, la metabolización por parte del hígado es muy rápida, desactivando en poco tiempo los efectos hormonales de ésta. Esta rápida metabolización ocurre debido a la vulnerabilidad de la molécula de progesterona a las enzimas, que rápidamente transforman la molécula [137]. En el caso de las progestinas, una vez administradas son parcialmente metabolizadas por las enzimas presentes en la flora y mucosa intestinal y, seguidamente, el hígado es el encargado de transformar tanto los progestágenos metabolizados como los libres, produciéndose una gran cantidad de metabolitos secundarios, principalmente compuestos glucurónidos y sulfatos que son excretados por la orina y las heces [144].

No es de extrañar que la principal fuente de contaminación de progestágenos al medio ambiente sean las EDARs, teniendo en cuenta los usos farmacológicos de este tipo de hormonas sexuales. Sin embargo, tal y

como se expuso en la Tabla 1.5., el estudio de la presencia de progestágenos en el medio está poco desarrollado en comparación con compuestos más estudiados y legislados como los estrógenos. Además, los progestágenos no son tan abundantes en las aguas residuales como los estrógenos o los andrógenos [145], lo que complica los estudios de presencia y degradación.

Diversos estudios como los llevados a cabo por Fan y colaboradores [145] o Vymazal y colaboradores [146] entre otros, han determinado que la degradación de progestágenos que se produce tanto en las EDAR tradicionales como en sistemas naturales basados en lagunaje, es bastante alta, con porcentajes de eliminación superiores al 70%, siendo los tratamientos anaeróbicos los más efectivos [145]. Los estudios de eliminación de progestágenos utilizando materiales adsorbentes también han sido muy satisfactorios, debido principalmente al carácter hidrófobo de este tipo de contaminante. De hecho, Grassi y colaboradores [147] determinaron una alta eliminación de progesterona utilizando carbón activado como material adsorbente. Sin embargo, otros tratamientos, como los basados en retención por membrana se han determinado como poco eficaces [148].

La eliminación de progestágenos de las aguas residuales es vital, puesto que su llegada al medio acuático produce múltiples efectos sobre los organismos que allí habitan. De hecho, desde los años 90 existen estudios toxicológicos que evidencian cambios en organismos acuáticos producidos por gestágenos [149]. Estudios más recientes han establecido que el levonorgestrel, una progestina ampliamente utilizada como fármaco, es el causante de producir cambios morfológicos tanto de

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anfibios jóvenes [150] como de peces [151] o de inhibir la producción de huevos en peces, incluso a concentraciones ambientales (en el nivel de  $\text{ng}\cdot\text{L}^{-1}$ ) [152]. Los efectos de otras progestinas así como la propia progesterona también han sido estudiados, encontrándose que son los responsables de cambios tanto en los sistemas reproductivos [152,153], como en el sistema nervioso central de algunos organismos [154] o, incluso, de cambios de comportamiento relacionados con la reproducción [155] de organismos acuáticos.

En la presente Tesis Doctoral se han estudiado cuatro progestágenos: la progesterona, cuyas características se han descrito anteriormente y tres progestinas: levonorgestrel, noretisterona y acetato de megestrol. Las estructuras de la progesterona y las tres progestinas analizadas se muestran en la Figura 1.12

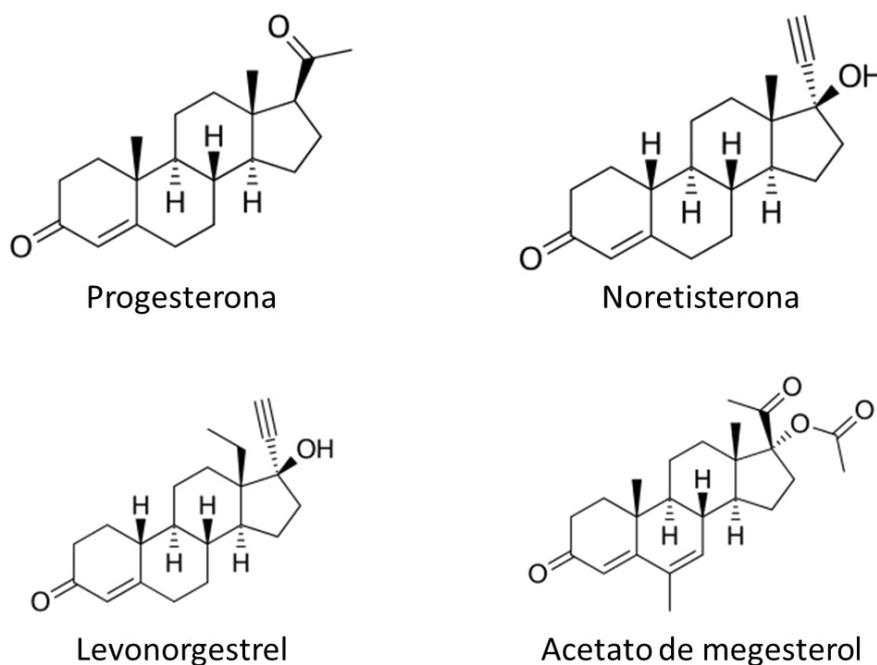


Figura 1.12. Estructuras moleculares de los progestágenos estudiados en la presente Tesis Doctoral

El levonorgestrel es una progestina sintética derivada de la 19-nortestosterona de tipo gonano, enantiómero levorrotatorio del norgestrel. Es el único isómero biológicamente activo del norgestrel, aunque en los fármacos basados en levonorgestrel suele existir una parte del enantiómero inerte. Teniendo en cuenta su estructura derivada de la 19-nortestosterona, presenta una leve actividad androgénica, nula actividad glucocorticoide [139], y una biodisponibilidad muy alta, en torno al 95% [142]. Una de las principales aplicaciones del levonorgestrel es como anticonceptivo de emergencia, mientras que en lo que respecta a su uso como anticonceptivo, se suele administrar junto al estrógeno sintético 17 $\alpha$ -etinilestradiol en una relación 5:1, mezcla que se ha estandarizado en farmacología [156]. Otro uso terapéutico es en terapias de reemplazo hormonal para aliviar los trastornos asociados a la menopausia, disminuyendo las posibilidades de hiperplasia [142].

Otra de las progestinas derivadas de la 19-nortestosterona, estudiada en la presente Tesis Doctoral, es la noretisterona. También conocida como noretindrona, difiere del levonorgestrel en que pertenece al subgrupo de los estranos y fue desarrollada y patentada en la década de 1950 [157] a partir de la molécula de estradiolmetileter. En pocos años comenzó a utilizarse en el tratamiento de trastornos ginecológicos y en la década de los 60 se empezó a utilizar en Estados Unidos como anticonceptivo [158]. La noretisterona no sólo se administra como molécula libre, sino que existen diversos profármacos basados en ella [159]. Una vez administrada, la noretisterona presenta una vida media bastante corta, produciéndose la metabolización a compuestos glucurónidos o sulfatos [160]. Al igual que ocurre con el levonorgestrel, la noretisterona produce efectos que dificultan la fecundación, haciendo que

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se utilice, por tanto, como anticonceptivo. Además, el uso de noretisterona tiene efectos en otros sistemas ya que produce una disminución del colesterol en mujeres que utilizan esta progestina en terapias de reemplazo hormonal tras la menopausia [161,162].

Finalmente, el acetato de megestrol es una progestina derivada de la molécula de progesterona, posee una estructura muy similar al acetato de medroxiprogesterona, y presenta una biodisponibilidad del 100% una vez administrado. Asimismo, se une casi exclusivamente a la albúmina plasmática, y sufre hidroxilación en los carbonos C<sub>21</sub>, C<sub>2</sub> y C<sub>6</sub> cuando es metabolizado [142]. Además de producir los efectos propios de un agente progestágeno, puede también actuar como glucocorticoide o como andrógeno [163], por lo que una de sus primeras utilidades fue el tratamiento de los cánceres de mama o endometrio, y posteriormente como estimulante del apetito [164]. Además, debido a sus efectos como glucocorticoide, puede producir, en pacientes expuestos a altas dosis, hipercortisolismo, diabetes o insuficiencia suprarrenal [165,166].

### 1.3.3.4. Glucocorticoides

A diferencia de los otros tres grupos desarrollados anteriormente, los glucocorticoides no se consideran hormonas sexuales y son segregados, junto a los mineralocorticoides en la corteza suprarrenal. Es importante destacar que existen dos tipos de corticoides, los de 19 carbonos, denominados androcorticoides y estrocorticoides, y los de 21 átomos de carbono. Estos corticoides de 21 átomos de carbono son los que realizan la actividad endocrina de la corteza suprarrenal y se dividen en glucocorticoides y mineralocorticoides [167]. Los mineralocorticoides

influyen en el transporte de electrolitos y agua en los tejidos, mientras que los glucocorticoides son los encargados de regular el metabolismo de proteínas, carbohidratos y lípidos. Además, debido a otros efectos asociados, los glucocorticoides se utilizan ampliamente en medicina, razón por la cual se han añadido al conjunto de hormonas estudiadas en la presente Tesis Doctoral.

Al igual que las hormonas sexuales, los glucocorticoides son derivados de la molécula del colesterol, el cual llega a la corteza suprarrenal donde pierde su cadena lateral y sufrirá varias reacciones que convertirán a la molécula en hidrocortisona (también denominada cortisol) [167]. Se consideran hormonas esteroideas porque presentan la estructura básica de ciclopentanoperhidrofenantreno y, aparte de esta estructura de 17 átomos de carbono, los glucocorticoides presentan grupos metilos unidos a los carbonos C<sub>10</sub> y C<sub>13</sub> y una cadena lateral de dos carbonos en el C<sub>17</sub>, lo que suma los 21 átomos de carbono característicos de este tipo de moléculas. Además, en el C<sub>17</sub> existe un grupo hidroxilo, que se coloca por debajo del plano de los anillos esteroides y un grupo cetónico unido al C<sub>3</sub>, el cual tiene una alta actividad biológica, al igual que la insaturación que presentan entre los carbonos C<sub>4</sub> y C<sub>5</sub>.

Los glucocorticoides presentan un mecanismo de acción similar al de las hormonas sexuales. Así, son capaces de traspasar la membrana celular unirse a los receptores específicos, los cuales se encuentran presentes en la mayoría de las células de los mamíferos [167], produciendo efectos no-genómicos, que se producen en segundos o pocos minutos, o efectos genómicos, los cuales tardan hasta varios días en desarrollarse en las células u órganos [168]. Los glucocorticoides tienen

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efectos en multitud de sistemas orgánicos como el aparato cardiovascular, los músculos, los huesos, la piel, el sistema nervioso o el linfático, entre muchos otros y son vitales en el metabolismo de los hidratos de carbono y los lípidos corporales [169]. También pueden tener efectos en la sangre, incrementando la hemoglobina y los glóbulos rojos, y en el sistema nervioso central, ya que pueden producir cambios de estado de ánimo y comportamiento, [170].

No obstante, el efecto más utilizado de los glucocorticoides es su capacidad antiinflamatoria, que se produce debido a que éstos interfieren con los mecanismos genéticos de la inflamación, haciendo que sean adecuados en el tratamiento de diversas enfermedades como asma, artritis reumatoide, colitis ulcerosa o enfermedad de Crohn [171]. De hecho, los glucocorticoides son mucho más efectivos que los antiinflamatorios no esteroideos, razón por la cual han llegado a ser los compuestos más comercializados como antiinflamatorios en todo el mundo [172,173].

Al ser administrados, los glucocorticoides pueden enlazarse a diferentes proteínas como la transcortina o la globulina de manera irregular, y además, presentan un metabolismo en dos pasos: en el primero se suelen añadir átomos de oxígeno o hidrógeno a la estructura de las hormonas, mientras que en la segunda se produce la conjugación con ácido glucurónico o sulfúrico, lo que hace que sólo entre el 1 y el 20% de los glucocorticoides se excrete en forma libre a través de orina y heces [174].

Teniendo en cuenta su uso eminentemente como fármaco y su forma de excreción, las estaciones depuradoras de aguas residuales se

establecen como fuente principal de emisión de glucocorticoides al medio acuático. El estudio de la presencia de glucocorticoides tanto en muestras de aguas residuales como en aguas superficiales es bastante reciente y limitado [175]. De hecho, Chang y colaboradores establecen su estudio de seis glucocorticoides en aguas residuales y superficiales realizado en 2007 como el primero [176], lo que evidencia la escasa información que se tiene sobre los glucocorticoides en el medio. A partir de este estudio se han realizado otros como el estudio cualitativo de aguas con actividad ligada a glucocorticoides llevado a cabo por Stavreva y colaboradores en 2012, donde establecían que más de la cuarta parte de las aguas estudiadas presentaban actividad glucocorticoide [175]. Paralelamente, también en 2012, Liu y colaboradores publicaron un estudio en el que evaluaban las concentraciones de cinco glucocorticoides, junto a otras hormonas esteroideas, en aguas residuales de dos EDARs, así como las eficacias de las tecnologías de depuración utilizadas en la eliminación de este tipo de compuestos. Estos autores encontraron que la concentración total de glucocorticoides en los influentes era la segunda más alta, tras la de andrógenos y seguida por las concentraciones totales de estrógenos y progestágenos, mientras que en el agua depurada la concentración total de glucocorticoides era, de media, la más baja. Además, establecieron eliminaciones de, prácticamente, el 100% y que el principal mecanismo de eliminación de este tipo de hormonas en los tratamientos de depuración es la degradación, ya que las concentraciones detectadas en los lodos de las EDARs fueron muy bajas o inexistentes [120].

Pese al gran espectro de terapias médicas en las que se utilizan glucocorticoides, no existe actualmente una legislación que limite o regule la presencia de glucocorticoides en el medio acuático. Sin embargo,

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debido al relativo efecto anabolizante que producen, los glucocorticoides han sido contemplados en regulaciones de dopaje en el deporte, estando prohibidas por cualquier vía de administración. [117]. Por otra parte, a nivel europeo, la Directiva 96/23/CE relativa a las medidas de control aplicables respecto de determinadas sustancias y sus residuos en los animales vivos y sus productos, determina que los glucocorticoides son “sustancias o productos no autorizados”, entendiéndose que su administración a animales (salvo en tratamientos veterinarios) está prohibida por la normativa comunitaria [58].

Sin embargo, aunque no se encuentren suficientemente regulados, los glucocorticoides son un riesgo para los ecosistemas acuáticos ya que pueden producir diversos efectos adversos en los organismos que los habitan. Entre otros, Nakayama y colaboradores determinaron que la exposición a glucocorticoides sintéticos a concentraciones ambientales producía un incremento de aminoácidos libres en el suero sanguíneo de carpas comunes (*Cyprinus carpio*) así como un incremento de los niveles de glucosa [177]. Sin embargo, este mismo autor en 2016 determinó que la susceptibilidad a la infección por bacterias de la carpa común no se ve afectada por la exposición de éstas a concentraciones ambientales relevantes de glucocorticoides [178]. Por otra parte, McNeil y colaboradores determinaron que, aunque exposiciones a concentraciones ambientales de prednisolona no producían efectos morfológicos en el desarrollo ocular del pez cebra (*Danio rerio*), sí que se apreciaban cambios conductuales asociados a la visión, como preferencia por áreas o ambientes oscuros [179]. Teniendo estos efectos en cuenta, algunos autores como Kugathas y Sumpter han determinado que los

glucocorticoides pueden tener efectos en organismos acuáticos a concentraciones muy bajas ( $100 \text{ ng}\cdot\text{L}^{-1}$ ) [180].

En la presente Tesis Doctoral se han analizado tres glucocorticoides, uno de ellos endógeno, la cortisona y los otros, sintéticos, la prednisona y la prednisolona (Figura 1.13).

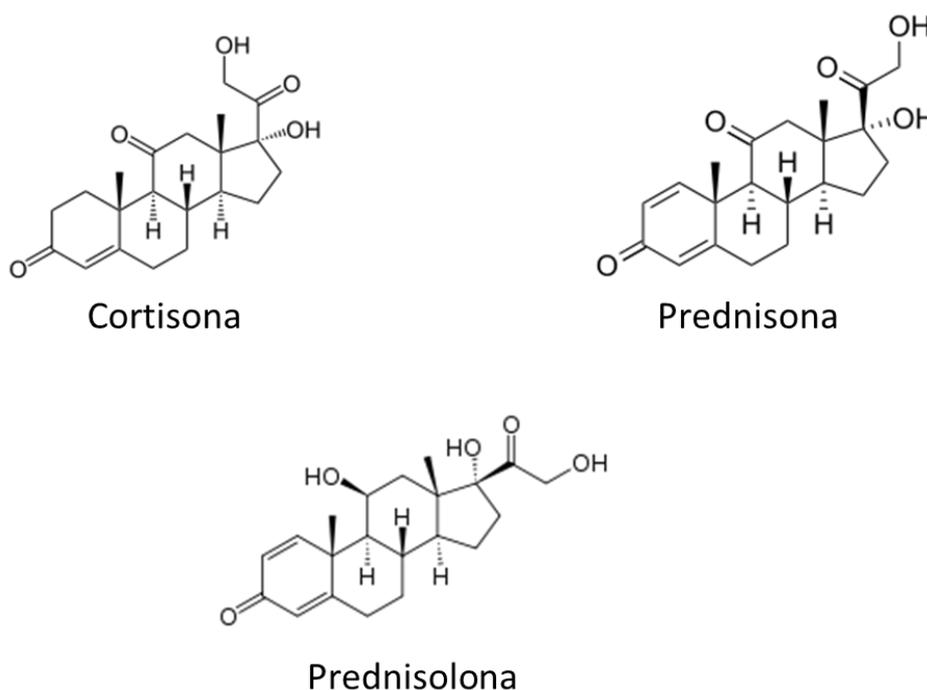


Figura 1.13. Estructuras moleculares de los glucocorticoides estudiados en la presente Tesis Doctoral

La cortisona es una hormona de tipo pregnano (21 átomos de carbono), estructuralmente muy parecida a la corticosterona y el cortisol. El cortisol es el principal precursor de cortisona, que se forma a partir de la oxidación del grupo hidroxilo del cortisol del carbono 11. Una vez formada, la cortisona puede sufrir diferentes metabolizaciones como reducción del doble enlace de los carbonos  $\text{C}_4$  y  $\text{C}_5$ , reducción del grupo cetónico del  $\text{C}_3$  o conjugación con ácido glucurónico [181]. La cortisona se

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considera un glucocorticoide de acción corta, debido a que su vida media es de 30 minutos y tiene una potencia glucocorticoide baja, muy similar a la del cortisol, sin embargo, al contrario que ocurre con otros glucocorticoides de potencia mayor, presenta una cierta actividad mineralocorticoide [181]. La cortisona ha sido ampliamente utilizada en medicina, debido a sus efectos en el organismo, desde hace más de 50 años, utilizándose en el tratamiento de insuficiencia de la corteza suprarrenal, enfermedades reumáticas, dermatológicas, oftalmológicas o alérgicas debido a su carácter antiinflamatorio [182].

Por su parte, la prednisona y la prednisolona son glucocorticoides sintéticos que presentan la particularidad de que pueden interconvertirse el uno en el otro. De hecho, la prednisolona es el compuesto activo de ambos, ya que es metabolito de la prednisona. Ambos compuestos presentan una marcada actividad glucocorticoide, cuatro veces superior a la del cortisol, no presentan actividad mineralocorticoide [181] y son absorbidos rápidamente tras ser administrados por vía oral, pues presentan una vida media de unas 3,5 horas para la prednisona y entre 2 y 3,5 horas, la prednisolona [183]. Sus usos son muy amplios pues se utilizan en el tratamiento de multitud de afecciones como alergias, trastornos dermatológicos, enfermedades que producen inflamación como la colitis o la artritis y en trastornos respiratorios. En lo que respecta a la prednisolona, la OMS ha indicado su uso en el tratamiento de procesos cancerígenos como leucemia o linfomas no-Hodgkin [184]. Sin embargo, su carácter inmunodepresor es uno de los efectos secundarios más importantes, sobre todo en pacientes con tratamientos de larga duración, aunque también pueden producir otros efectos como osteoporosis

prematura, debido a que afectan a componentes básicos en el mantenimiento óseo [184,185].

### **1.4. Metodologías de extracción y determinación de hormonas esteroideas en muestras medioambientales**

Debido a que las hormonas esteroideas se encuentran en el medio a niveles traza o ultratrazas, el análisis de éstas en el medio ambiente se convierte en un verdadero reto para los analistas. El proceso analítico deberá comenzar realizando técnicas de extracción y preconcentración que permitan por un lado separar los analitos de interés de la muestra en la que se encuentran y eliminar a su vez las interferencias que puedan afectar a la correcta determinación y, por otra, preconcentrar los analitos extraídos, lo cual aumentará la sensibilidad del análisis.

Sin embargo, en la mayoría de los casos, antes incluso de la extracción de las hormonas esteroideas de la matriz en la que se encuentran, se hace necesaria la aplicación de ciertos pretratamientos que permitan bien el correcto análisis de la muestra o su almacenamiento en condiciones que no produzcan una degradación de los analitos que contiene. En este sentido, se ha comprobado que es necesaria la adición de algún tipo de disolvente o agente para prevenir la degradación de las hormonas por parte de las bacterias presentes en la muestra. Esta adición de disolventes es indispensable en el caso de matrices con gran carga biológica como aguas o lodos procedentes de EDARs, ya que la acción microbiana es más acelerada que en matrices más limpias como aguas superficiales o sedimentos [186]. Se ha evidenciado que la inhibición de la actividad bacteriana se puede llevar a cabo acidificando la muestra con ácido clorhídrico, sulfúrico o acético hasta pH inferior a 3,5 o añadiendo

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una pequeña cantidad de formaldehído (normalmente un 0,1% en volumen), en el caso de muestras líquidas [186–190]. Además, la mayoría de autores conserva la muestra en frío [186–189], mientras que en el caso de muestras sólidas y biológicas se realiza una liofilización para eliminar el agua presente en la muestra, lo cual inhibe también la actividad bacteriana para, posteriormente, congelar la muestra hasta su análisis [191–193].

El pretratamiento de las muestras prosigue con la filtración o tamizado, dependiendo de si se dispone de una muestra líquida o sólida. En el caso de la filtración, lo habitual es usar filtros con un tamaño de poro de entre 0,45 y 1,5  $\mu\text{m}$  de materiales como fibra de vidrio [186–188] o membrana [194,195]. Por su parte, las muestras sólidas una vez homogeneizadas y liofilizadas, suelen pulverizarse y tamizarse, para utilizar en el análisis la fracción obtenida, que suele estar comprendida entre 0,5 y 1,5 mm [191,196]. Una vez llevados a cabo todos estos pretratamientos se pueden realizar los métodos de extracción y preconcentración necesarios para llevar a cabo el análisis.

### 1.4.1. Métodos de extracción y preconcentración

#### 1.4.1.1. Muestras líquidas

Los avances que se han llevado a cabo en las últimas décadas en el campo de la química analítica han permitido el desarrollo de metodologías de detección y determinación cada vez más selectivas y sensibles. Sin embargo, pese a estos avances, dichas técnicas requieren un paso previo de extracción y preconcentración ya que en muchas ocasiones las muestras son muy complejas y presentan una alta cantidad de

interferencias, como ocurre en las aguas residuales. En matrices más limpias la presencia de interferencias no es tan preocupante, sin embargo, las concentraciones de hormonas sufren una gran dilución debido a que llegan a grandes masas de aguas como ríos, lagos, mares u océanos, por lo que estos tratamientos de extracción y preconcentración son también necesarios. La elección, por tanto, del método idóneo para realizar la extracción y preconcentración dependerá de las características de la muestra que se desee analizar.

La técnica de extracción tradicional es la extracción líquido-líquido (LLE), la cual se basa en la transferencia de los analitos de la matriz a una fase acuosa orgánica, la cual se lleva a cabo mediante sucesivas etapas de agitación. Aunque existen estudios que han utilizado esta técnica en la extracción de hormonas esteroideas en muestras ambientales [197] o biológicas [198], debido a su sencillez y aceptables recuperaciones, no se ha desarrollado ya que se utilizan grandes cantidades de disolventes orgánicos, los cuales suelen ser tóxicos. Estas desventajas han provocado que la LLE ha quedado relegada en detrimento de técnicas más sostenibles y próximas a los principios de la “química verde” [199].

Una de estas técnicas sostenibles es la extracción en fase sólida (SPE, *Solid Phase Extraction*), la cual se ha convertido en las últimas décadas en la técnica de extracción y preconcentración más utilizada y estandarizada. De hecho, la SPE es utilizada en protocolos de análisis de diversas administraciones como la US-EPA o la Comisión Europea [200,201]. La extracción en fase sólida está basada en la retención selectiva de los analitos en una fase adsorbente y su posterior elución, utilizando un disolvente adecuado. Los analitos presentan una mayor

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afinidad por la fase adsorbente que por el disolvente en el que se encuentran disueltos por lo que, cuando la muestra pasa a través del medio adsorbente, los analitos quedan retenidos en su superficie, mientras que los otros componentes de la muestra no retenidos pasan a través de él. Seguidamente se realiza un paso de lavado para eluir las posibles interferencias que hayan quedado retenidas sobre el adsorbente con un disolvente que no eluya los analitos de interés. Finalmente, en el último paso se utiliza un disolvente, normalmente orgánico, para eluir los analitos de interés retenidos. Dependiendo del adsorbente utilizado, así como del disolvente orgánico que se utilice en este procedimiento se pueden obtener extracciones realmente selectivas, por lo que el estudio y optimización de los pasos anteriormente descritos, así como la elección de fases adsorbentes y eluyentes adecuados es vital para obtener recuperaciones lo más altas posibles. La Figura 1.14. muestra la secuencia de pasos que se deben realizar en la extracción en fase sólida.

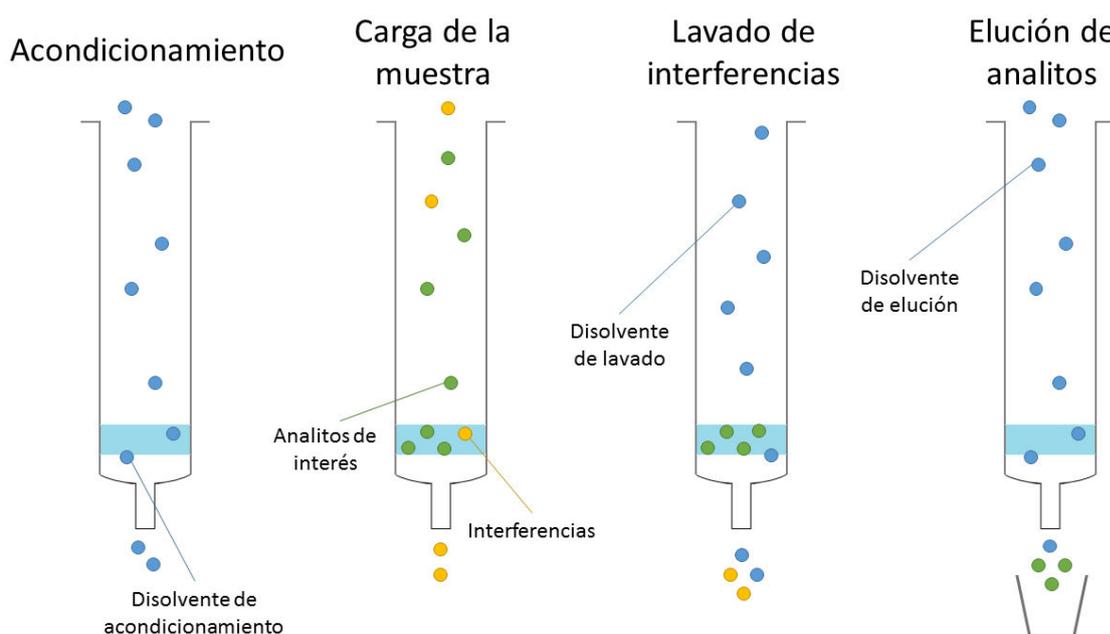


Figura 1.14. Etapas en la extracción en fase sólida.

Al utilizar un pequeño volumen de disolvente orgánico para eluir las muestras adsorbidas en el cartucho, se consigue, no solo extraer los analitos en un volumen de muestra manejable, sino, preconcentrar éstos, lo que redundará en métodos de análisis muchos más sensibles, capaces de extraer concentraciones de contaminantes en el rango de los  $\text{ng}\cdot\text{L}^{-1}$ , o incluso menores. Además, la SPE no sólo sirve para la extracción y preconcentración de analitos, sino que también es útil en la limpieza de muestras, eliminando interferencias, para fraccionar los analitos extraídos de una muestra realizando una sucesión de lavados, cada uno de ellos adecuado para un tipo de analito o, incluso, para el almacenamiento de analitos extraídos de muestras en las que son inestables o puedan degradarse [202]. Debido a la estandarización de esta técnica como método de rutina existen multitud de adsorbentes poliméricos comerciales utilizados como fase estacionaria, entre los que se encuentran adsorbentes para extracciones en fase normal, en fase reversa o por intercambio iónico. Entre los adsorbentes clásicos encontramos aquellos basados en óxido de silicio y unidos a diferentes grupos funcionales como grupos etilo ( $\text{C}_2$ ), octilo ( $\text{C}_8$ ), octadecilo ( $\text{C}_{18}$ ) o fenilo ( $\text{C}_6$ ) en el caso de extracciones en fase reversa y grupos cianopropilo (CN) o aminopropilo ( $\text{NH}_2$ ) en fase normal [203]. Pese a la alta aplicabilidad de los adsorbentes basados en sílice, éstos presentan el inconveniente de que el rango de pH que soportan es limitado y que los residuos de silanol ligados a este tipo de compuestos pueden convertirse en una interferencia muy importante a la hora del análisis [204]. Más recientemente se han desarrollado medios adsorbentes basados en polímeros porosos, principalmente copolímeros de estireno-divinilbenceno (PS-DVB), que presentan una mayor estabilidad al pH de la muestra, no producen residuos de silanol y debido a su balance

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hidrófilo-lipófilo tienen un alto grado de retención para sustancias de diferentes naturalezas [204–206]. En el caso de las hormonas esteroideas los adsorbentes más utilizados son los de sílice unida a grupo C<sub>18</sub>, así como los poliméricos de tipo PS-DVB, los cuales se suelen utilizar en métodos de análisis multiresiduo para la extracción de diversos tipos de fármacos.

Las tendencias actuales de la química analítica pasan por la automatización, así como el empleo de técnicas cada vez más selectivas y específicas, lo cual permite obtener límites de detección y cuantificación cada vez más bajos. En este sentido, estas tendencias han sido aplicadas en la SPE, desarrollándose métodos de extracción en fase sólida automatizados o, polímeros adsorbentes cada vez más selectivos. Aparte de la automatización del método, en los últimos 5-7 años se ha desarrollado una modalidad de SPE conocida como SPE online que permite el acoplamiento de un sistema automatizado de extracción en fase sólida a un sistema de separación y determinación de los analitos. Los sistemas on-line, por tanto, se han revelado como una potente mejora ya que no sólo se consiguen minimizar los errores asociados a la manipulación de las muestras, sino que las cantidades de disolventes orgánicos utilizadas son 1 o 2 órdenes de magnitud inferiores a la SPE tradicional.

Por otra parte, la otra innovación relacionada con la SPE es el desarrollo de nuevos adsorbentes, cada vez más específicos para un tipo de contaminante. En este aspecto, se han desarrollado los polímeros impresos molecularmente (MIPs, *molecular imprinted polymers*) que consisten en materiales sintéticos creados utilizando la molécula o estructura que se quiera extraer como “plantilla”. Utilizando estas

moléculas o estructuras como base, se utilizan uno o dos monómeros, conocidos como monómeros funcionales, los cuales son polimerizados en presencia de uno o dos monómeros denominados entrecruzadores. Los monómeros funcionales, una vez polimerizados, conforman una estructura que se mantiene, gracias a la polimerización, una vez se ha retirado la “molécula plantilla”. Así, el polímero resultante presenta una memoria molecular que le permite retener moléculas iguales o muy similares a las de las cavidades que se han formado en su estructura [207–209]. Además, otras ventajas de este tipo de adsorbentes son su tolerancia a altos rangos de pH, temperaturas y disolventes orgánicos [210]. En el caso de las hormonas esteroideas, es lógico pensar que se utilice la estructura básica de ciclopentanoperhidrofenantreno para realizar la impresión molecular de estos polímeros, obteniéndose así un medio adsorbente de alta especificidad y selectividad para la extracción de hormonas esteroideas presentes en muestras medioambientales [211,212].

Aparte de la extracción en fase sólida y teniendo en cuenta las tendencias actuales de miniaturización, en las últimas décadas se han desarrollado multitud de técnicas de microextracción para hormonas que buscan conseguir altos factores de preconcentración para poder medir las concentraciones traza ambientales y minimizar, a su vez, la cantidad de disolventes orgánicos asociados a estas tareas. La primera de estas técnicas y más desarrollada hasta el momento es la microextracción en fase sólida (SPME, *solid-phase microextraction*). La SPME fue desarrollada por Arthur y Pawliszyn a principios de los años 90 [213] y se basa en el uso de una fibra de sílice fundida recubierta de material adsorbente, la cual suele ser de unos centenares de micras de grosor. Este pequeño tamaño

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permite incorporar la fibra al interior de una jeringa, haciéndola transportable y permitiendo hacer la extracción *in situ*. Una vez realizada la extracción, la fibra puede ser guardada nuevamente en la jeringa hasta el momento de la desorción, la cual puede ser térmica o líquida, dependiendo del instrumento de análisis que se vaya a utilizar, así como de las características de los analitos extraídos. La estandarización de dicha técnica ha hecho que se desarrollen fibras de SPME recubiertas de multitud de polímeros adsorbentes, aunque las más utilizadas, en el caso de las hormonas esteroideas son las de poliacrilato o carbowax-divinilbenceno [214–216]. No obstante, también existen estudios que han sintetizado fibras específicas que presentan una capacidad de extracción similar a la de las fibras comerciales, como las fibras monolíticas desarrolladas por Liao y colaboradores [217].

Otra de las técnicas utilizadas para hormonas esteroideas ha sido la extracción asistida por barras agitadoras (SBSE, *stir-bar sorptive extraction*), la cual fue desarrollada casi una década después de la SPME por Baltussen y colaboradores [218]. En este tipo de extracción, ésta se realiza sobre una barrita agitadora recubierta de un polímero (normalmente polidimetilsiloxano, PDMS) y la posterior desorción puede llevarse a cabo de manera térmica o líquida. La SBSE presenta algunas ventajas sobre la SPME, como una mayor superficie adsorbente y la capacidad de reutilizar la barra agitadora más veces que las fibras de SPME [219] aunque su principal desventaja estriba en el hecho de que no existen muchas alternativas al recubrimiento de PDMS para realizar la extracción [220]. Diversos autores han desarrollado métodos de extracción basados en SBSE para hormonas esteroideas tanto en muestras

de agua [221,222] como en muestras líquidas biológicas como orina [223,224], obteniendo buenas selectividades y factores de recuperación.

Aproximadamente al mismo tiempo que el desarrollo de la SBSE, se comenzaron a desarrollar metodologías que buscaban la miniaturización de la clásica extracción líquido-líquido. Estas técnicas solventan una de las limitaciones de la SPME y la SBSE ya que los dispositivos utilizados no pertenecen a ninguna marca comercial, por lo que la flexibilidad es mayor [225]. Este aspecto ha hecho que se desarrollen multitud de métodos de microextracción líquido-líquido en los que difieren aspectos como el dispositivo utilizado o la forma en la que se produce la extracción de los compuestos. Para el caso de las hormonas esteroideas, de la multitud de métodos de microextracción desarrollados, se pueden encontrar estudios basados en microextracción líquido-líquido dispersiva (DLLME, *dispersive liquid-liquid microextraction*) [226], microextracción en fase líquida por fibra hueca (HF-LPME, *hollow fiber liquid-phase microextraction*) para extraer estrógenos [227,228] o microextracción en fase líquida por gota sólida (SD-LPME, *solid drop liquid phase microextraction*) [229].

También se han desarrollado técnicas miniaturizadas en fase sólida para la determinación de hormonas como la microextracción mediante película fina (TFME, *thin film microextraction*) [230], la cual es una mejora de la SPME y, más recientemente, la extracción por adsorción sobre tejidos químicamente modificados (FPSE, *fabric phase sorptive extraction*). Esta última técnica ha sido desarrollada por Kabir y colaboradores en el año 2014 [231] y se basa en el uso de un tejido que sirve como soporte a un sorbente polimérico sobre el que se adsorben los analitos presentes en la muestra. Actualmente esta técnica ha sido utilizada para la

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determinación de diferentes tipos de compuestos como surfactantes, filtros solares, medicamentos o herbicidas en muestras líquidas biológicas y ambientales [232–235]. Los beneficios de esta técnica de microextracción frente a otras son su versatilidad, pues permite utilizar gran cantidad de medios adsorbentes diferentes, dependiendo de la especie química que se quiera extraer; la gran superficie de adsorción, superior a la de otras técnicas como la SPME o la SBSE; los tiempos relativamente cortos de análisis así como la facilidad de manipulación de la muestra y los medios extractantes [232]. No obstante, al igual que ocurre con el resto de métodos de extracción, se hace necesaria la optimización de las diferentes variables involucradas en la técnica, como tiempo de extracción, volumen de muestra o naturaleza y volumen de eluyente, para conseguir los mayores rendimientos y factores de recuperación.

### **1.4.1.2. Muestras sólidas y biológicas**

Las muestras sólidas como sedimentos o lodos de EDARs, así como las biológicas como tejido animal, necesitan someterse a otro tipo de métodos de extracción para separar la matriz de los analitos de interés. En las extracciones de matrices sólidas se utiliza un disolvente con propiedades afines a las del analito que se quiera extraer, el cual se pone en contacto con la matriz haciendo que el contaminante pase de estar adsorbido sobre la matriz sólida a estar disuelto en el disolvente orgánico. Tras este paso de extracción puede ser necesaria la purificación del extracto para eliminar restos de la matriz, mediante filtración o evaporación de los extractos, lo cual produciría además una preconcentración de los analitos. En este sentido se han desarrollado

diferentes técnicas de extracción para muestras sólidas, siendo la primera de ellas la extracción *Soxhlet*. Aunque en la actualidad existen métodos y procedimientos recomendados por organizaciones de control de la calidad del medio ambiente que utilizan esta técnica para la extracción de hormonas de muestras sólidas [236], la extracción *Soxhlet* presenta importantes desventajas como los largos tiempos de extracción, que pueden llegar hasta las 48 horas, así como las grandes cantidades de disolvente orgánico necesarias. Este último inconveniente, además plantea otro asociado, que es la necesidad de realizar una etapa de evaporación para concentrar los extractos.

Como alternativa a la extracción *Soxhlet* se desarrolló la extracción asistida por ultrasonidos (UAE, *ultrasound-assisted extraction*) que utiliza la energía proporcionada por éstos para favorecer el paso de los analitos adsorbidos sobre la fase sólida a la fase líquida. El fenómeno que se produce al aplicar ultrasonidos a una mezcla de sólido y disolvente se conoce como cavitación y consiste en la formación de pequeñas burbujas que crecen hasta explotar provocando incrementos locales de presión y temperatura. Pese a que la cavitación producida por los ultrasonidos favorece la extracción, lo que permite que los tiempos de extracción sean más cortos que en la extracción *Soxhlet*, la diferencia de energías producida hace que los resultados sean menos reproducibles [237]. Sin embargo, las ventajas que presenta esta técnica son el uso de menores cantidades de disolventes que la extracción *Soxhlet*, la posibilidad de realizar extracciones a temperaturas inferiores a la temperatura de ebullición del disolvente extractante o la posibilidad de controlar la temperatura para conseguir rendimientos mayores. Estos beneficios han provocado que la extracción asistida por ultrasonidos haya desplazado a la

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extracción *Soxhlet* como método de extracción en muestras sólidas. En la actualidad existen diversos estudios que utilizan la UAE para realizar la extracción de hormonas de matrices sólidas como lodos de estaciones depuradoras, sólidos en suspensión presentes en aguas residuales o sedimentos de ecosistemas acuáticos [55,238–240].

Tal y como ocurre con las técnicas de extracción para muestras líquidas, las tendencias actuales se centran en la miniaturización de técnicas que permitan análisis en tiempos más cortos y con menor gasto de reactivos. Por ello se han desarrollado técnicas muy robustas y eficientes como la extracción con líquidos presurizados (PLE, *presurized liquid extraction*), la extracción por fluidos supercríticos (SFE, *supercritical fluid extraction*) o la extracción asistida por microondas (MAE, *microwave-assisted extraction*).

La PLE emplea tiempos aún más cortos que la UAE para realizar la extracción la cual se realiza utilizando una pequeña parte de disolvente que se pone en contacto con la muestra en un dispositivo donde se pueden aplicar temperaturas relativamente altas y altas presiones. Estas condiciones de presión y temperatura permiten utilizar disolventes a temperaturas superiores a su punto de ebullición que producirán la ruptura de las uniones entre la matriz y el analito, extrayendo éste [241]. Con un fundamento similar, también se ha desarrollado la extracción por fluidos supercríticos en la que se utiliza como agente extractante un fluido en condiciones supercríticas, es decir a presiones y temperaturas en las que su comportamiento tiene características de líquido y gas, a la vez [242]. Este tipo de extracción permite realizar extracciones muy eficientes debido a que la solubilidad y difusividad de los fluidos utilizados es mayor,

y además se puede realizar una extracción prácticamente total del analito al usar extractante continuamente renovado o “fresco”. Otros puntos a favor de esta técnica son, por un lado, la posibilidad de acoplarlo al sistema de detección (lo que se conoce como modalidad online) y por otro que se utilizan de fluidos supercríticos como el dióxido de carbono, que son baratos, relativamente ecológicos y no tóxicos [242,243]. Sin embargo, el principal inconveniente tanto de la PLE como de la SFE es el coste de los aparatos necesarios para realizar la extracción, razón por la cual su uso no se ha estandarizado. En el caso de la extracción de hormonas sexuales y glucocorticoides, varios estudios han utilizado PLE o SFE en muestras como sedimentos, sólidos de estaciones depuradoras o matrices comestibles [244–248].

Para superar la principal desventaja de la PLE y la SFE que radica en los altos costes asociados a los instrumentos capaces de llevar a cabo estas técnicas, se desarrolló de manera paralela la extracción asistida por microondas. Las microondas se consideran radiaciones no ionizantes con una frecuencia comprendida entre 0,3 y 300 GHz, aunque la práctica totalidad de hornos microondas usan microondas de 2,45 GHz de frecuencia, produciendo calentamiento de dos maneras, mediante rotación dipolar y conducción iónica, según la naturaleza de las moléculas presentes en la muestra [249]. La principal ventaja del calentamiento mediante microondas es que la energía incide directamente en las moléculas de la muestra, lo que hace que el calentamiento sea de dentro hacia afuera, al contrario que ocurre en el calentamiento convencional. Esto se consigue ya que se utilizan recipientes “transparentes” para las microondas como el vidrio, el cuarzo o el teflón [250], los cuales presentan constantes dieléctricas muy bajas. Estas características convierten a la

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MAE es una técnica muy selectiva, pues el calentamiento puede producirse sólo en la muestra o también en el disolvente, dependiendo de las características de los analitos que se quieran analizar. En lo que respecta a la instrumentación, ésta es muy sencilla pues sólo es necesario un microondas (los cuales son análogos a los dispositivos domésticos) y un carrusel que permite la colocación de los vasos reactores. Estos vasos pueden ser abiertos, en los que la extracción se realiza a temperatura ambiente, o cerrados, lo que permite trabajar de manera presurizada en condiciones en las que los disolventes pueden encontrarse en forma líquida pero a temperaturas superiores a sus temperaturas de ebullición [251]. En el desarrollo de un método de MAE existen diversas variables que deben ser optimizadas para cada analito o tipo de analitos, así como para cada tipo de matriz. Estas variables son cantidad de matriz, tipo y volumen de disolvente, potencia de microondas y tiempo de extracción. La cantidad de matriz utilizada no suele ser muy alta, mientras que como disolvente se suele usar un disolvente orgánico o mezcla de ellos. En lo que respecta a la potencia de microondas y el tiempo de extracción, también es muy importante su estudio detallado ya que pasado un límite, se puede producir degradación de la muestra o de los analitos, extracción de sustancias no deseadas o evaporación del disolvente [252]. A pesar de las ventajas de la técnica, como su selectividad, el uso de pequeños volúmenes de disolvente o los cortos tiempos de extracción, presenta también inconvenientes como la necesidad de realizar un paso de limpieza para los extractos o la dificultad para acoplarla a un sistema de detección. La extracción asistida por microondas ha sido ampliamente utilizada para el análisis de compuestos orgánicos como productos naturales o contaminantes emergentes en muestras ambientales como sedimentos,

sólidos de depuradora, alimentos o matrices biológicas [253–258]. En el caso de las hormonas existen pocos estudios en los que se haya desarrollado un método basado en MAE para la extracción de todas las familias de éstas, siendo lo más habitual el estudio de estrógenos, progestágenos o andrógenos, por separado o en combinación de algunas de ellas [259–261].

Finalmente, en el campo de las matrices biológicas se ha popularizado en los últimos años el uso de la extracción “QuEChERS”, la cual recibe su nombre al realizar el acrónimo de las palabras *quick, easy, cheap, effective, rugged, safe* (en español: rápido, fácil, barato, eficaz, robusto y seguro). Este tipo de extracción, desarrollado en 2003 por Anastassiades y colaboradores, se diseñó, en un principio, para la extracción de pesticidas en matrices alimenticias complejas como frutas y verduras [262]. El método se basa en una extracción realizada a micro escala, ya que el volumen de disolvente usado puede ser de varios mililitros, utilizando además diferentes sales para favorecer la extracción, seguida de la purificación del extracto mediante una extracción en fase sólida dispersiva que se realiza combinando sulfato de magnesio y uno o varios sorbentes [263,264]. Aunque la extracción “QuEChERS” se diseñó para matrices biológicas, además de haber sido estudiada en éstas [265,266], también se han conseguido resultados satisfactorios en la extracción de hormonas de otras matrices como suelos o sedimentos [267,268].

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### 1.4.2. Métodos de separación y determinación

Tras realizar la extracción de las hormonas esteroideas de la matriz en la que se encuentran, llega el momento de proceder a su separación y posterior determinación. Para estos fines, las técnicas existentes pueden ser divididas en dos grandes grupos que son las técnicas biológicas, basadas en inmunoensayos, y las cromatográficas, tanto de gases como líquidas. En el caso de los inmunoensayos, encontramos radioinmunoensayos (RIA, *radioimmunoassay*) e inmunoensayos directos como la técnica ELISA (*Enzyme-Linked ImmunoSorbent Assay*), los cuales se han utilizado principalmente en el ámbito clínico, por lo que raramente se han utilizado para la determinación en muestras ambientales [197]. A pesar de su robustez y facilidad de realización, los métodos basados en inmunoensayos son poco específicos y muy dependientes de que exista efecto matriz, por lo que su fiabilidad no es muy alta [269]. Además, los inmunoensayos tienden a sobreestimar las concentraciones de hormonas esteroideas y no permiten la medida de muchas hormonas a la vez, por lo que su uso ha ido progresivamente disminuyendo, en detrimento de las técnicas basadas en cromatografía [270].

De los dos tipos de técnicas cromatográficas, la cromatografía de gases (GC, *gas chromatography*) fue la más utilizada hasta finales de los años 90 [197]. Los métodos de cromatografía de gases acoplados a espectrometría de masas (GC-MS) o a espectrometría de masas de triple cuadrupolo (GC-MS/MS) fueron los más desarrollados para la determinación de hormonas, debido a los bajos límites de detección obtenidos. Estas técnicas presentan ventajas como mejores resoluciones cromatográficas o mejor sensibilidad que la cromatografía líquida a

compuestos derivados como los esteroides, o a metabolitos de las hormonas, por lo que su uso en metabolómica es bastante interesante [271]. Sin embargo, la cromatografía de gases presenta algunas desventajas insalvables que han hecho que no se considere como técnica única en el análisis de hormonas en muestras ambientales. Estas desventajas son que requiere largos procesos de preparación de la muestra, la imposibilidad de automatizar el proceso o la incapacidad de la detección de compuestos hormonales conjugados debido a su muy baja volatilidad [271]. Otro aspecto que a veces provoca la desestimación de la GC como técnica de determinación es la necesidad de derivatización de las hormonas extraídas. Este paso se hace necesario para conseguir aumentar la volatilidad y estabilidad térmica de las hormonas, lo cual produce un aumento de la sensibilidad del método, pero, como contrapartida, introduce un paso en el tratamiento de muestra bastante tedioso, que provoca que el tiempo total de análisis aumente considerablemente [272]. Además, en el caso particular de las hormonas conjugadas es necesaria la hidrólisis de éstas, seguida de una derivatización, por lo que los límites de detección obtenidos no son tan bajos como con otras técnicas de determinación, debido a los rendimientos, tanto del paso de hidrólisis como del de extracción del analito [273]. En este sentido, Croley y colaboradores han comparado la sensibilidad de métodos basados en cromatografía de gases y líquida acoplada a espectrometría de masas y han determinado que el orden de sensibilidad de las técnicas es el siguiente: LC-MS/MS > GC-MS/MS > LC-MS [274].

### 1.4.2.1. Metodologías basadas en cromatografía líquida para la determinación de hormonas esteroideas en sistemas medioambientales acuáticos.

Los factores vistos en el apartado anterior, unidos al fuerte desarrollo que ha tenido la cromatografía líquida de alta resolución (HPLC) en los últimos quince años, han provocado que ésta sea la más utilizada en la actualidad para la separación y determinación de hormonas en muestras medioambientales, tanto líquidas como sólidas, por lo que existen una gran cantidad de trabajos en esta área [271,275]. La razón principal del desarrollo de metodologías analíticas basadas en cromatografía líquida para determinar hormonas estriba en la baja volatilidad de las hormonas esteroideas, así como el hecho de que ciertas hormonas como el estriol no pueden determinarse en cromatografía de gases. En la actualidad se han desarrollado métodos de separación y determinación de hormonas basados en cromatografía líquida con detectores de diodo de array, de fluorescencia, en el caso específico de los estrógenos, y sobre todo de espectrometría de masas, tanto de cuadrupolo simple (MS), como de triple cuadrupolo o en tándem (MS/MS)

En el caso particular de los métodos basados en cromatografía líquida de alta resolución con detección de espectrometría de masas, se ha conseguido identificar y cuantificar hormonas muy similares estructuralmente, de idéntico peso molecular o, incluso, isómeros de una misma hormona ya que se unen en un solo método la gran capacidad de separación de la cromatografía líquida y la extraordinaria especificidad de la espectrometría de masas. Por esta razón, esta técnica está llamada a ser técnica de referencia en el ámbito del análisis de hormonas esteroideas,

así como de ser elegida por las diferentes Administraciones como técnica de rutina. Además, las mejoras de ambas técnicas, como el desarrollo de la cromatografía líquida de ultra resolución (UHPLC) o los sistemas de espectrometría de masas capaces de determinar metabolitos y productos derivados como los detectores de espectrometría de masas de tiempo de vuelo (TOF, *time of flight*) o de trampa de iones (*Ion-trap*) demuestran la tendencia de la comunidad científica internacional de desarrollar esta potente herramienta de análisis químico y medioambiental.

Teniendo esto en cuenta, en el siguiente trabajo se ha hecho una revisión bibliográfica del estado del arte en cuanto a los diversos métodos desarrollados en los últimos diez años, para la determinación de hormonas esteroideas en los diferentes compartimentos medioambientales, lo que incluye matrices líquidas, sólidas y biológicas, en los que se utilizan cromatografía líquida acoplada a distintos sistemas de detección de espectrometría de masas, y con diversos procedimientos previos de extracción y preconcentración de las muestras.

Esto muestra la preocupación actual que existe en la comunidad científica internacional por la presencia de las hormonas esteroideas en los diferentes compartimentos medioambientales, y el enorme potencial que presenta la cromatografía líquida acoplada a la espectrometría de masas, como técnica instrumental en el campo de la química analítica y el análisis ambiental.

El trabajo ha sido publicado en la revista *Trends in Environmental Analytical Chemistry* 3-4 (2014), 14 – 27, la cual publica revisiones bibliográficas en el campo de la química analítica y medioambiental.





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### Review

## Liquid chromatography methodologies for the determination of steroid hormones in aquatic environmental systems



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#### ABSTRACT

Steroid hormones are a diverse group of natural and synthetic compounds. Their wide use in human and veterinary medicine results in their continual introduction into the environment. In recent years, environmental concern over steroids that act as endocrine disruptors has increased because of their adverse effects on organisms or their progeny. Moreover, as these compounds are not totally removed from sewage in wastewater treatment plants, they can reach the aquatic environment and persist due to their physicochemical characteristics.

For this reason, a major trend in analytical chemistry is the development of rapid and efficient procedures for the extraction, determination and quantification of steroid hormones in environmental samples. Over the past few decades, the significant expansion of liquid chromatography technology utilizing mass spectrometry detection has led to applications with increased selectivity and sensitivity. Optimized extraction and microextraction techniques combined to these liquid chromatography techniques have lowered detection and quantification limits to the  $\text{ng L}^{-1}$ – $\mu\text{g L}^{-1}$  range, which is the concentration of steroid hormones in liquid, solid and biota samples.

In this paper, the state-of-the-art techniques for the analysis of steroid hormones focused mainly in based liquid chromatography methods in liquid and aquatic solid and biota samples are reviewed. Handling, storage, extraction and detection methodologies are reviewed and compared for all families of steroid hormones.

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*Abbreviations:* ACN, acetonitrile; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photoionization; ASE, accelerate solvent extraction; BEH, bridge ethyl hybrid; CPE, cloud point extraction; DAD, diode array detection; DLLME, dispersive liquid–liquid microextraction; DMAE, dynamic microwave assisted extraction; EDCs, endocrine disruptor compounds; ESI, electrospray ionization; GC, gas chromatography; GH/IGF-I, growth hormone and insulin-like growth factor I; H<sub>2</sub>SO<sub>4</sub>, sulphuric acid; HCl, hydrochloric acid; LC, liquid chromatography; LLE, liquid–liquid extraction; MAE, microwave assisted extraction; MeOH, methanol; MS, mass spectrometer; MSPD, matrix solid phase dispersion; PLE, pressurised liquid extraction; QuEChERS, quick easy cheap effective rugged safe; SBSE, stir bar sorptive extraction; SFO, solidification of a floating drop; SPE, solid phase extraction; UAE, ultrasonic assisted extraction; UHPLC, ultra-high performance liquid chromatography; USEPA, United States Environmental Protection Agency; UV, ultraviolet–visible; WHO, World Health Organization; WWTP, wastewater treatment plant.

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## 1. Introduction

The quality of life on earth is intimately linked to the quality of the environment. In many countries, marine, terrestrial and aerial environmental problems; the increasing demand for water and; the constant discovery of potentially dangerous pollutants are serious issues. This situation necessitates research in all areas related to the protection of human health and the sustainable use of natural resources. The presence of emergent pollutants in the environment is one of the main issues concerning organizations committed to public and environmental health, such as the World Health Organization (WHO), the United States Environmental Protection Agency (USEPA) and the European Commission [1].

Many current pollution issues stem from intermittent or continuous leakage of chemical substances into the environment. The toxicity of these compounds to flora, fauna and humans has been demonstrated in several recent works [2,3]. Key amongst environmental contaminants are the endocrine disruptor compounds (EDCs). EDCs are a large group of natural and synthetic compounds that are defined as exogenous substances or mixtures that alter functions of the endocrine system and consequently cause adverse health effects in an intact organism, its progeny, or subpopulations [4,5].

Steroid hormones are a wide group of biologically active endocrine disruptor compounds that control many functions of endocrine systems. The main characteristic of steroid hormones is their cyclopentane-perhydro-phenanthrene structure, also known as sterane, derived from cholesterol. Partial removal of the cholesterol side chain results in the 21-carbon progestogens or gestagens and corticosteroids. The complete removal of the side chain provides 19-carbon products, called androgens. Finally, additional removal of the C-19 methyl group by aromatization produces 18-carbon compounds, known as estrogens. The names, acronyms and structures of the hormones reviewed in this paper are listed in Fig. 1a–d.

Steroid hormones can be divided into five subgroups based on structural characteristics: estrogens, androgens, progestogens, glucocorticoids and mineralcorticoids [6]. Within these groups, there are both natural and synthetic hormones. Natural steroids are mainly synthesized in the sex organs, adrenal glands and placenta.

Estrogens are female sex hormones, which are also present in males, and they control many physiological activities, such as growth and reproduction. The global consumption of estrogens has increased exponentially over the last 50 years due to their use as contraceptives, menopausal therapies, as treatment for some human cancers and as veterinary growth promoters. Estrogens are now considered to be the most prescribed drugs in the world [7]. Estrogens are subject to metabolic hydroxylation, oxidation and methylation and undergo biotransformation to sulphates and glucuronides. However, a significant amount of estrogens are excreted from the organism as free estrogens or conjugates. For example, after excretion, 17 $\beta$ -estradiol is rapidly oxidised to estrone, which can be transformed into estriol. Conversely, 17  $\alpha$ -ethynylestradiol is excreted as a conjugate in most cases [8]. There are several synthetic estrogens, such as diethylstilbestrol, that are not steroid hormones because they do not present the steroid structure. However, their characteristics and behaviors are similar to steroid estrogens and they are often studied together.

Progestogens, also known as gestagens, present a 21-carbon pregnane structure. They are called progestins because of their role in maintaining pregnancy, but they are also present in other phases of oestrous and menstrual cycles. As with estrogens, there are natural and synthetic progestogens. Gestagens are used in the treatment of several cancers and some menstrual disorders, but their main use is as an anticonceptive, sometimes in conjunction with estrogens. The observation that progesterone suppresses ovulation during pregnancy led to its development as a contraceptive. To minimize progesterone-related complications in contraceptive drugs, progestins were developed; norethisterone, was one of the first oral contraceptives sold in the United States [9].

Androgens are male sex hormones, and their main function is the control of male sexual characteristics. The three main androgens are testosterone, androsterone and dihydrotestosterone. Many analogues of these compounds have been developed as therapeutics and as anabolic substances, promoting muscular growth [10]. Their use as anabolic compounds has been highly controversial due to associated health problems. In children, anabolic compounds can result in growth retardation and precocious puberty. In veterinary medicine androgens, such as testosterone or trenbolone acetate, have been widely used as growth promoters in cattle farming [11].

Glucocorticoids are a group of hormones excreted by the adrenal cortex (cortisol, cortisone) and are synthetically produced (dexamethasone, triamcinolone). They control the energy supply through gluconeogenesis and suppress stress-related inflammation and infection [12], making them useful in the treatment of several allergies, asthma, and arthritis or polymyalgia rheumatica [13]. Natural glucocorticoids are excreted as free glucocorticoids or conjugated glucocorticoids in both urine and dregs. In veterinary medicine, they are used as androgens to restore muscle strength and size.

Hormones can be of endogenous or exogenous origin. Biosynthetic steroid hormones are endogenous, while xenobiotic or exogenous steroids are foreign compounds, which are naturally or synthetically produced. Steroid hormones excreted by humans reach the aquatic environment daily via sewage systems, and several authors have stated that municipal wastewaters are the main source of contamination of aquatic environments [14,15]. This contamination is due to the stability, resistance to microbial degradation, and bio availability of hormonal compounds [16]. Moreover, industrial wastewaters are a source of hormonal contamination as well. Microbial degradation of phytosterols to androgens has been proposed as a natural source of androgenic steroids, which could explain elevated concentrations of androgenic activity downstream from paper mills [17].

Sumpter and Jobling's [18] finding in 1995, that municipal wastewater discharges induce physiological responses in fish, which is indicative of exposure to estrogens, has led to an increase in studies on the effects of hormonal compounds in biota [19]. Low concentrations (50 ng L<sup>-1</sup>) of ethynylestradiol, for example, have been linked to diminished reproduction in zebra fish [20], while changes in the production of vitellogenin in trout [21] has been observed at concentrations of 1 ng L<sup>-1</sup> of 17 $\beta$ -estradiol or its equivalents. Also, environmental concentrations of ethynylestradiol have been found to interfere with the GH/IGF-I system in bony fish [22].

The studies of the effects in biota of other hormonal compound families are scarce relative to oestrogen-related studies. Database searches of Web of Science and CSA Illumina for publications over the past 20 years produced 4.4–4.9 times the number of references associated with “estrogens and environmental” versus “androgens and environmental” [23]. The first publication regarding the androgenic activity of effluents [24] came 9 years after the Sumpter and Jobling’s study. More recently, the change in anal fin morphometrics in female mosquitofish produced by androstenedione [25], and degeneration in the liver and kidney produced by injections of 11-ketotestosterone [26] in adult Sockeye salmon have been reported.

Finally, ecotoxicological effects of gestagens and glucocorticoids in aquatic organisms have been studied. Long-term cortisol treatment inhibits locomotion, aggressive behavior and competitive ability in rainbow trout [27,28]. In the case of progestins, norgestrel, for example, inhibited the post-breeding regression of secondary sexual characteristics as well as the resumption of spermatogenesis of male three-spined stickleback, [29] while progesterone increased ex vivo testosterone production in fathead minnow [30].

Given these facts, many methods of extraction, preconcentration and determination of steroid hormones have been developed to determine the presence of these compounds in various matrices.

In last 10 years, many authors have carried out some reviews in which are exposed the state-of-the-art of the analysis of steroids in the environment. For example, Ying et al. [8] reviewed the occurrence of some estrogens in environmental waters and animal waste, while Hamid and Eskicioglu [31] did the same in sludge matrices. Aris et al. [32] linked the concentrations that obtained other authors with the effects in exposed biota, but only with an estrogen, the 17 $\alpha$ -ethynylestradiol. Other authors studied two families of steroids, as Kozłowska-Tylingo et al. [33] that reviewed different chromatographic methods for estrogens and progestogens and compare it with bioanalytical methods or Noppe et al. [34] that reviewed novel analytical methods for the determination of estrogens and androgens in edible matrices. In this paper, we update the previous reviews of extraction and preconcentration techniques developed over the last 15 years to determine and quantify all the families of steroid hormonal compounds in environmental water and solid samples and aquatic biota. To describe all the bibliography about steroids in the environment, we have restricted the analysis to liquid chromatography.

2. Applicable regulations

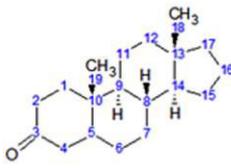
Considering the emission sources and the varied effects of steroid hormones on aquatic organisms, it is logical that they have

(a)

Estrogens	Compound	Acronym	C3	C16	C17
	Estrone	E1			=O
	Estrone-3-sulfate	E1-3S	-O-SO <sub>3</sub> <sup>-</sup>		=O
	Estrone-3-glucuronide	E1-3G			=O
	17 $\alpha$ -estradiol	$\alpha$ E2			
	17 $\beta$ -estradiol	E2			
	17 $\beta$ -estradiol-17-acetate	E2-17A			
	17 $\beta$ -estradiol-3 $\beta$ -glucuronide	E2-3G			-OH
	17 $\beta$ -estradiol-17-glucuronide	E2-17G			
	17 $\beta$ -estradiol-3-sulfate	E2-3S	-O-SO <sub>3</sub> <sup>-</sup>		-OH
	Estriol	E3			
	Estriol-3-glucuronide	E3-3G			
	Estriol-16-glucuronide	E3-16G			
	Estriol-3-sulfate	E3-3S	-O-SO <sub>3</sub> <sup>-</sup>		
	17 $\alpha$ -ethynylestradiol	EE			

Fig. 1. Names, acronyms and structures of the hormones reviewed. (a) : Estrogens, (b) : Androgens, (c) : Progestogens, (d) : Glucocorticoids.

(b)

Androgens	Compound	Acronym	C1-C2	C3	C4-C5	C5	C5-C6	C10	C17
	19-nor-4-androstene-3,17-dione	NAD			==			-H	=O
	1,4-androstadienedione	ANDD	==		==				=O
	5-androstane-3,17-diol	5-A-3,17-DIOL		..... OH		..... H			..... OH
	5 $\alpha$ -dihydrotestosterone	DHT				..... H			..... OH
	Androstenedione	ADD			==				=O
	Androsterone	AND		..... OH		..... H			=O
	Boldione	BLDN	==		==				=O
	Boldenone	BOL	==		==				..... OH
	Dehydroepiandrosterone	DHEA		..... OH			==		=O
	Epiandrosterone	EPA		..... OH		..... H			=O
	Epitestosterone	EPITES			==				..... OH
	Ethynyltestosterone	ETES			==				..... OH
	Etiocholanolone	ETIO		..... OH		..... H			=O
	Methyltestosterone	METES			==				..... OH ..... CH <sub>3</sub>
	Nandrolone	NAN			==			..... H	..... OH
	Testosterone	TES			==				..... OH
Testosterone-17-proprionate	PTS			==				..... -O-CO-C <sub>2</sub> H <sub>5</sub>	
	Compound	Acronym	C1-C2	C4-C5	C9-C10	C10	C11	C11-C12	C17
	Trenbolone	TBL		==	==			==	..... OH
	17 $\alpha$ -Trenbolone	17 $\alpha$ -TBL		==	==			==	..... OH
	11-Ketotestosterone	KTS		==		..... CH <sub>3</sub>	=O		..... OH

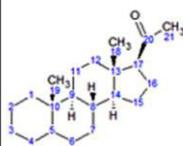
Progestogens	Compound	Acronym	C3	C4-C5	C5	C6	C6-C7	C10	C11	C15-C16	C17	C18	C20	C21	
	17 $\alpha$ ,20 $\beta$ -dihydroxy-4-pregnen-3-one	DPO	=O	==							..... OH		..... OH		
	17 $\alpha$ -hydroxyprogesterone	17-HPT	=O	==							..... OH				
	21 $\alpha$ -hydroxyprogesterone	21-HPT	=O	==										..... OH	
	5 $\beta$ -pregnan-3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -triol	5 $\beta$ -3 $\alpha$ ,17 $\alpha$ ,20 $\beta$ -P		..... OH		..... H						..... OH			
	6 $\alpha$ -methyl-hydroxyprogesterone	MHPT	=O	==		..... CH <sub>3</sub>			-OH						
	Ethinodiol diacetate	ETH-DIA	..... O	==				..... H			..... O				
	Drospirenone	DROS	=O	==			-CH <sub>2</sub> -			-CH <sub>2</sub> -	..... O				
	Medroxyprogesterone	MEPRO	=O	==		..... H					..... OH				
	Medroxyprogesterone acetate	MPA	=O	==		..... H					..... O				
	Megestrol	MEG	=O	==		-CH <sub>3</sub>	==				..... OH				
	Megestrol acetate	MGA	=O	==		-CH <sub>3</sub>	==				..... O				
	Norethisterone	NORET	=O	==				..... H			..... OH				
	Norgestrel	NORG	=O	==				..... H			..... OH	-CH <sub>3</sub>			
Progesterone	PRO	=O	==							..... H					

Fig. 1. (Continued)

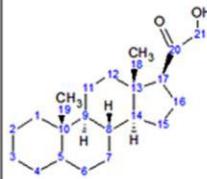
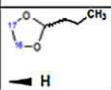
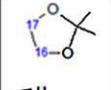
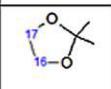
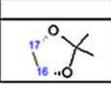
Glucocorticoids	Compound	Acronym	C1-C2	C3	C4-C5	C6	C9	C11	C16	C16-C17	C17
	6 $\alpha$ -methylprednisolone	6-MPRNL	==	= O	==	◀ H					◀ OH
	Betamethasone	BMS	==	= O	==		◀ F	◀ OH	◀ CH <sub>3</sub>		◀ OH
	Budesonide	BDS	==	= O	==			◀ OH			
	Cortisol	CORS									◀ OH
	Corticosterone	COR		= O	==			◀ OH			
	Cortisone	CORT		= O	==			= O			◀ OH
	Desonide	DESO	==	= O	==			◀ OH			
	Dexamethasone	DEXA	==	= O	==		◀ F		◀ CH <sub>3</sub>		◀ OH
	Flumethasone	FMS	==	= O	==	◀ H - F	◀ F	◀ OH	◀ CH <sub>3</sub>		◀ OH
	Flucinolone acetonide	FCN-ACE	==	= O	==	◀ H - F	◀ F	◀ OH			
	Prednisone	PRD	==	= O	==			= O			◀ OH
	Prednisolone	PRNL	==	= O	==			◀ OH			◀ OH
	Triamcinolone	TRIAM	==	= O	==			◀ F	◀ OH	◀ OH	◀ OH
Triamcinolone acetonide	TRIAM-ACE	==	= O	==		◀ F	◀ OH				

Fig. 1. (Continued)

been included in recent Directives. The use of these compounds in livestock is regulated in the European Union by 96/22/EC directive [35], and recently, the European Commission proposed the 17 $\beta$ -estradiol and the 17 $\alpha$ -ethynylestradiol to include in the last modification of the Directive 2000/60/EC [36]. In this draft, environmental quality standards (EQS) expressed as an annual average value (AA-EQS) were proposed for both compounds in inland surface waters (rivers, lakes and related artificial or heavily modified water bodies) and other surface waters. The proposed concentrations, which must not be exceeded, were 4. 10<sup>-4</sup> and 8.10<sup>-5</sup>  $\mu\text{g L}^{-1}$  (17 $\beta$ -estradiol) and 3.5.10<sup>-5</sup> and 7.10<sup>-6</sup>  $\mu\text{g L}^{-1}$  (17 $\alpha$ -ethynylestradiol) for inland surface waters and other surface waters, respectively.

Finally, the Directive 2013/39/EU, amending the Directive 2000/60/EU and 2008/105/EC as regards priority substances in the field of water policy [37], did not consider as priority either of this two compounds, but it included them in the first watch list to gather monitoring data for the purpose of facilitating the determination of appropriate measures to address the risk posed by these substances. The watch will be updated every 24 months thereafter, allowing the inclusion of one new compound in each update up to a maximum number of 14. With this normative, the Member States must monitor the 17 $\beta$ -estradiol and the 17 $\alpha$ -ethynylestradiol at selected representative monitoring stations over at least 12-month period. For this reason is necessary to obtain more data about the presence of these analytes in the environment and the possible effects that they can exert in order to elucidate if in the future they must be included in the priority compounds list.

### 3. Sample preparation and extraction process

Environmental samples are challenging to analytical chemists because they are highly complex matrices requiring several sample preparation steps, such as filtration, purification, and extraction, or

preconcentration. These processes are, in most cases, time-consuming, but they are essential for successful subsequent analytical determination. Specific processes vary greatly for different matrices. At times, samples with similar characteristics can require different processing. Below we have listed procedures according to matrix type: liquid, solid or biota.

#### 3.1. Liquid samples

##### 3.1.1. Sample handling and storage

Samples are usually collected in glass bottles rinsed with organic solvents and water [38–48]. Depending of the extraction process, the sample volume needed ranges from a few millilitres for microextraction techniques or on-line solid phase extraction (SPE) [39,47,49–52] to thousands of millilitres for traditional SPE [14,23,41–43,48,53–55]. Matrix characteristics also impact the choice of sample volume. Required volumes of influent samples are lower than effluent samples, and these are lower than samples of environmental waters, such as river waters, ground waters or tap water [41,44,45,53,55].

Laboratory samples are generally refrigerated (4°C). Often, solvents or additives are included as preservatives. To inhibit bacterial activity, sample pH is usually adjusted to values between 2 and 3 with hydrochloric acid (HCl) [14,23,44,45], sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) [38,54] or formic acid (HCOOH) [42]. Wang et al. [50] reports the use formaldehyde at a concentration of 1% to preserve the samples until the extraction process.

##### 3.1.2. Filtration and extraction processes

Filtration of the sample is an indispensable pre-extraction step because environmental waters, especially wastewater samples, have large amounts of suspended solids that can affect the extraction and preconcentration process. Many authors have reported filtration using membrane filters or glass-fibre filters.

**Table 1**  
Extraction and determination methods of steroid hormones in liquid samples.

Hormones studied	Matrix	Sample volume (mL)	Extraction process	Analytical column	Detection method	LOD (ng L <sup>-1</sup> )	Recovery (%)	References
Estrogens: E2, E3, E1, EE	River water	2000	SPE	Zorbax Eclipse XDB-C18	LC-UV	240–510	78–101	[14]
Estrogens: E1, E2, E3, EE Androgens: TES, AND, EPA, DHEA	WWTP effluent and pond water	650–1000	SPE disk	Acquity BEH C18	UHPLC-ESI-MS/MS	E1, E2, E3, EE: 1200–45000TES: 640 AND, EPA, DHEA: 100,000	Estrogens: 66–120 Androgens: 64 – 120	[23]
Estrogens: E2, E3, E1, MES, EE,DES Progesterons: PRO, ETH-DIA, NORG, NORET	WWTP influent and effluent water	500	SPE	Lichrospher 100 RP-18	HPLC-DAD-MS	Estrogens: 25–500Progesterons: 2–10	Estrogens: 56–93 Progesterons: 105–113	[33]
Estrogens: E1, E2, E3, EE Progesterons: PRO, MIEPRO, NORET, NORG	WWTP influent, effluent and superficial water	1	On-line SPE	Hypersild GOLD	LC-APPI-MS/MS	Estrogens: 3–50 Progesterons: 2–7	85–110	[34]
Estrogens: E3–3 S, E3–16 G, E2–17 G, E1–3 G, E2–3 S, E3, E1–3 S, E2, EE, E1, DES	River, lake and canal water	500	SPE	Purospher STAR RP-18e	LC-ESI-MS/MS	Estrogens: 0.1–7 Progesterons: 0.4–3.5	Estrogens: 14–60 Progesterons: 81–108	[35]
Progesterons: PRO, NORET, NORG Androgens: ADD, TES, NAD, AND, EPA, METES, NAN	WWTP influent, effluent and river water	500–2000	SPE	Acquity UPLC BEH C18	UHPLC-ESI-MS/MS	Androgens: 0.1–50 Progesterons: 0.01–1.2	78–100	[36]
Progesterons: PRO, 17-HPT, 21-HPT, MHPT, DPO, NORET, NORG, MPA, MGA	Lake, well and ground water	1000	Automated SPE	Zorbax eclipse XDB C18	LC-ESI-MS/MS	Estrogens: 0.03–0.2 Androgens: 0.01–3.6 Progesterons: 0.01–0.8	95–100	[37]
Estrogens: αE2, E2, E1, E3, EE Androgens: TES, EPTES, ADD, ETIO, DHT, 5-A-3-17-DIOL, AND, EPA Progesterons: PRO, DROS, NORG, NORET, MEPRO, MEG	Estuary and river water	2000	Automated SPE	ODS-100S C18	LC-APPI-MS/MS	Estrogens: 0.4–12 Androgens: 0.1–7	Estrogens: 74–107 Androgens: 90–105	[38]
Estrogens: E1, E2, E3, EE Androgens: TES, ADD, AND, EPA, DHEA, DHT, DHEA-3S	WWTP influent, effluent and river water	100–500	SPE	Kromasil 100C18	HPLC-ESI-MS/MS	15–100	46–87	[39,40]
Estrogens: DES, E1, E2, αE2, E3, EE, E2–17 A, E1–3 S, E2–3 S, E1–3 G, E2–17 G	WWTP influent, effluent and river water	250	SPE	Pursuit XRS ultra-C18	HPLC-ESI-MS/MS	0.9–4	83–96	[41]
Estrogens: E1, E2, E3, EE Androgens: TES Progesterons: MGA, NORG	WWTP influent and effluent water	5	On-line SPE	Acquity UPLC BEH C18	UHPLC-ESI-MS/MS	0.5–1.3	74–105	[42]
Estrogens: E1, E2, E3, EE, DES Androgens: TES Progesterons: MGA, NORG	WWTP influent and effluent water	250 mL	SPE	Acquity UPLC BEH C18	UHPLC-ESI-MS/MS	0.2–9.3	51–91	[43]
Estrogens: E1, E2, E3, EE Progesterons: PRO Androgens: BLDN, TBL, ADD, NAN, BOL, TES, EPA, METES, 4-OHA, DHT,	Tap and river water	5	DLLME-SFO	Acquity BEH phenyl column	UHPLC-UV	800–3100	87–116	[44]
Estrogens: E1, E2, E3 Progesterons: PRO Androgens: BLDN, TBL, ADD, NAN, BOL, TES, EPA, METES, 4-OHA, DHT,	WWTP effluent and river water	10	CPE	Intertsil ODS-C18	HPLC-UV	Estrogens: 230–320 Progesterons: 5000	82–99	[45]
Estrogens: E1, E2, αE2, EE, DES, MES Progesterons: NORET, NORG, PRO Glucocorticoids: PRD, PRNL, CORT, COR3, DEXA, 6-MPRNL	Wellwater	30	SRSE	120 OctaDecil-silica-A	HPLC-DAD	Estrogens: 600–1000 Progesterons: 300–600	11–100	[46]
Estrogens: E1, E2, E3, EE, DES Androgens: BLDN, TBL, ADD, NAN, BOL, TES, EPA, METES, 4-OHA, DHT,	WWTP influent, effluent and river water	70–2000	SPE	Acquity UPLC BEH C18	UHPLC-ESI-MS/MS	0.01–0.2	73–99	[47]
Estrogens: E1, E2, EE, DES Androgens: BLDN, TBL, ADD, NAN, BOL, TES, EPA, METES, 4-OHA, DHT,	WWTP influent, effluent and river water	1000	SPE	Zorbax SB-C18	RLC-ESI-MS/MS	Estrogens: 0.1–1.4 Androgens: 0.01–0.9 Progesterons: 0.02–0.4 Glucocorticoids: 0.03–0.5	Estrogens: 73–123 Androgens: 64–164 Progesterons: 54–142 Glucocorticoids: 76–192	[48]

Table 1 (Continued)

Hormones studied	Matrix	Sample volume (mL)	Extraction process	Analytical column	Detection method	LOD (ng L <sup>-1</sup> )	Recovery (%)	References
AND Progestogens: NORET, ETES, NORG, MEPRO, PRO Glucocorticoids: PRD, CORT, CORS, PRNL, DEXA Estrogens: DES, E1, αE2, E2, EE Androgens: ADD, AND, EPA, NAD, NAN, METES, TBL, TES Progestogens: DPO, 17-HPT, 21-HPT, MHPT, MGA, MPA, NORG, NORET, PRO Estrogens: E1, E2, E3, αE2, DES, DIEN	WWTP influent, effluent and river water Tap and river water	70–2000 100	SPE SPE	Acquity UPLC BEH C18 Ascentis express phenyl-hexyl Supelcosil ABZ+PLUS	UHPLC-ESI-MS/MS UHPLC-APCI-MS/MS	Estrogens: 0.02–1 Androgens: 0.03–40 Progestogens: 0.02–2 4.2–9.8	78–100 47–98	[49] [50]
Androgens: BOL, EPITES, METES, NORTES Progestogens: PRO Glucocorticoids: TRIAM, CORS, DEXA, FLUM, PRNL, TRIAM-ACE	River water	100–1000	SPE	Supelcosil ABZ+PLUS	LC-APCI-MS/MS	Androgens: 0.3–0.9 Progestogens: 0.1–0.7 0.4 Glucocorticoids: 58–110	Androgens: 80–96 Progestogens: 102 Glucocorticoids: 58–110	[51]

most commonly used were 0.45 μm [14,38–40,42,44–46,49,50,56], 0.65 μm [47,48], 0.70 μm [41,54] and 1.2 μm [55].

The extraction and preconcentration process is necessary due to the low concentration of steroid hormones in environmental samples. The main extraction and preconcentration technique for aqueous environmental samples is SPE and its variants (automated SPE or on-line SPE) [14,23,38–57]. There are many different sorbents used in SPE methods, for example, polymers with hydrophilic-lipophilic balance, such as OASIS HLB used in wastewater [41,44,45,47,53–55] and superficial water samples [41,44,53–55,57], C<sub>18</sub> cartridges or disks [23,38,39,42,46,48,52] used in the analysis of estrogens, progestogens and androgens in wastewater and environmental water samples, polymeric sorbents [14,40], graphitized non-porous carbon sorbent used in the analysis of superficial waters [43], or in recent years, molecularly imprinted polymers [56] for the analysis of estrogens in tap and river water.

The extraction step used is not specific to the family of steroid being isolated. In many cases, steroid hormones from different families were extracted with a single extraction and preconcentration step [23,38–43,47,48,50,51,54,55,57]. An additional purification step to remove interfering substances in the sample is often carried out. These purification steps are usually used in influent and effluent samples and river water samples collected upstream and downstream of a wastewater treatment plant (WWTP). The main sorbents used are silica gel cartridges [41,54] and Florisil cartridges [43]. Sometimes a combination of the two sorbents is used. Chang et al. [55] first used silica gel cartridges to purify an extract for the determination of androgens and progestogens and then used a Florisil cartridge to purify the extract for the determination of estrogens.

Common solvents are used for extraction of steroid hormones. In SPE, methanol and acetonitrile, separately or in combination, are routinely employed to extract analytes from the aqueous sample [14,23,38–40,42,44–48,56,57]. Ethyl acetate [41,53–55], methylene chloride [43,57] and hexane [40] have also been used. The volume of extract is 1–10 mL in most cases, and many authors add a drying step to improve preconcentration. Using SPE, the recoveries are satisfactory, greater than 60% in most cases [14,23,38,39,41–57]. The choice of sorbent does not depend on the compound family, but some authors noticed that recovery of glucuronide and sulphate conjugated estrogens is lower than that of the free estrogens studied [40,43–45].

In recent years, many authors have reported the use of microextraction techniques to extract and preconcentrate estrogens and progestogens using dispersive liquid-liquid microextraction with solidification of a floating drop (DLLME-SFO) [49], cloud point extraction (CPE) [50] or stir bar sorptive extraction (SBSE) [51] with satisfactory recoveries. The solvents used in microextraction are the same for SBSE (methanol and acetonitrile) [49], 1-undecanol, and TritonX-114 for DLLME-SFO and CPE, respectively [50,51]. Using new microextraction techniques, the recoveries were between 82 and 116% for DLLME-SFO and CPE, and variable recoveries (11–46% for estrogens and 20–100% for progestogens) were obtained for the SBSE technique.

Table 1 summarizes the determination methods of the steroid hormones in liquid samples.

### 3.2. Solid and aquatic biota samples

#### 3.3.1. Sample handling and storing

Samples weights are usually less than 5 g for each analysis of sediment, sludge and aquatic biota samples. Only Froehner et al. [58] used more than 5 g of sample in the extraction of estrogens from mangrove sediments. After collection, solid and biota

**Table 2**  
Extraction and determination methods of the steroid hormones in solid and biota samples.

Solid samples								
Hormones studied	Matrix	Sample weight (g)	Extraction process	Analytical column	Detection method	LOD( $\text{ng} \cdot \text{g}^{-1}$ )	Recovery(%)	Reference
Estrogens: E1, E2, EE, DES Androgens: BLDN, TBL, ADD, NAN, BOL, TES, EPA, METES, 4-OHA, DHT, AND Proggestogens: NORET, ETES, NORG, MEPRO, PRO Glucocorticoids: PRD, CORT, CORS, PRDNL, DEXA	Wastewater sludge	0.5	UAE	Zorbax SB-C18	UHPLC-ESI-MS/MS	Estrogens: 0.1–1.0 Androgens: 0.1–0.7 Proggestogens: 0.4–1.9 Glucocorticoids: 0.6–2.1	Estrogens: 69–103 Androgens: 62–122 Proggestogens: 83–137 Glucocorticoids: 66–116	[48]
Estrogens: E1, E2, E3, EE	Mangrove sediment	20	UAE	ODS Hipersil C18	HPLC-UV	na	na	[52]
Glucocorticoids: BMS, CORS, CORT, DEXA, FMS, MPNL, PRNL, PRD, TRIAM-ACE	Wastewater sludge	1	PLE	Zorbax Eclipse XDB-C18	UHPLC-ESI-MS/MS	1–5	8–73	[53]
Estrogens: E1, E2, E3, EE	Wastewater sludge	1	PLE	Purospher STAR-RP-18e	LC-ESI-MS/MS	1–3	91–132	[54]
Estrogens: E2, E3, EE	Wastewater sludge	1	MAE	Pursuit XRs Ultra-C18	LC-ESI-MS/MS	0.9–1.5	72–102	[56]
Estrogens: E1, E2, E3, EE	Wastewater sludge	5	UAE	Atlantis dC18	LC-ESI-MS/MS			[57]
Estrogens: E1 Androgens: TES, ADD Proggestogens: NORET, NORG, PRO Glucocorticoids: DESO, BDS, TRIAM-ACE, FCN-ACE, BMS	Soil	5	PLE	Zorbax Eclipse XDB C18	HPLC-ESI-MS/MS	Estrogens: 0.9 Androgens: 0.1–0.3 Glucocorticoids: 0.1–2.8	Estrogens: 62 Androgens: 72–73 Proggestogens: 67–87 Glucocorticoids: 43–101	[58]
Estrogens: E1, E2, $\alpha$ E2, E3, EE Androgens: TES, KTS, 17 $\alpha$ -TBL, TBL, ADD, AND, ANDD, EPITES Proggestogens: 17-HPT, MLGA, PRO	Sediment and soil	3	MAE	HyPURITY C18	HPLC-APPI-MS/MS	Estrogens: 0.2–1.9 Androgens: 0.1–0.3 Proggestogens: 0.2–0.3	Estrogens: 62–186 Androgens: 52–121 Proggestogens: 51–85	[59]
Estrogens: E1, E2, E3, EE	Sediment	1	MSPD	Acquity BEH C18	UHPLC-ESI-MS/MS	0.07–0.11	89–102	[60]
Biota samples								
Hormones studied	Matrix	Sample weight (g)	Extraction process	Analytical column	Detection method	LOD( $\text{ng} \cdot \text{g}^{-1}$ )	Recovery(%)	Reference
Estrogens: E1, E2, E3, EE, E1–3S Proggestogens: PRO, NORG	Fish	0.5	QuEChERS	Acquity BEH C18	UHPLC-ESI-MS/MS	E1, EE, E1–3S: 0.01–0.81 E2, E3: 2.0–3.1 PRO, NORG: 0.3–0.6	Estrogens: 45–103 Proggestogens: 59–90	[55]
Estrogens: E1, E2, E3, EE	Fish, clam	1	MSPD	Acquity BEH C18	UHPLC-ESI-MS/MS	0.05–0.22	80–110	[60]
Androgens: TES, ADD Proggestogens: 5 $\beta$ -3 $\alpha$ , 17 $\alpha$ , 20 $\beta$ -P, DPO, 17-HPT, PRO	Ovarian follicles of perch	5	LLE	$\mu$ Bondapak C18	HPLC-UV	na	na	[61]
Estrogens: E3, $\alpha$ E2, E1 Androgens: NAN, TES Proggestogens: 17-HPT, MEPRO, PRO Glucocorticoids: COR	Shrimp, squid and fish tissue	3	DMAE	Zorbax eclipse C18	HPLC-ESI-MS/MS	0.03–0.15	79–94	[62]
Estrogens: E1, E2, EE, E3 Androgens: EPITES, NAN, METES, PTS Proggestogens: MEPRO, PRO	Fish muscle	5	UAE	Supelco discovery C18	LC-ESI-MS/MS	0.06–0.22	64–83	[63]
Estrogens: E1, E2, E3, EE, MES, DES	Mussel	5	UAE	Luna C8	HPLC-ESI-MS	0.3–5	44–77	[64]

samples are treated to eliminate the water that they contain. Many authors used freeze drying techniques to eliminate the water in sediments [58], sludge [54,59,60] or tissue [61]. Other authors air-dried the samples over several hours [62–64] or used wet sediments [65,66] or wet biota samples [66–68] in their

extractions. Practically all authors kept their solid or biota samples at  $-18$  or  $-20$  °C until the extraction process. [60–63,65]. Additives were not used to inhibit microbial activity, except as reported by Liu et al. [54] who added sodium azide to sludge samples to preserve them. Xu et al. [69] centrifuged fish tissues with acetic acid buffer and added an enzyme solution to release the steroids

**Table 3**  
Concentration of hormones in liquid, solid and biota samples.

Location	Matrix	Hormones studied	Concentrations (min-max)	Reference
Montreal (Canada)	WWTP influent, effluent and superficial water	Estrogens: E1, E2, E3, EE Progestogens: PRO, MEPRO, NORET, NORG	Influent samples Estrogens: E3, E2, EE: 75–243 ng L <sup>-1</sup> Progestogens: NORET, NORG, MEPRO: 5–205 ng L <sup>-1</sup> Effluent samples Estrogens: E2: 90 ng L <sup>-1</sup> Progestogens: NORET, NORG: 30–50 ng L <sup>-1</sup> River samples Estrogens: E2: 8–9 ng L <sup>-1</sup>	[34]
Rio de Janeiro State (Brazil)	River, lake and canal water	Estrogens: E3–3 S, E3–16 G, E2–17 G, E1–3 G, E2–3 S, E3, E1–3 S, E2, EE, E1, DES Progestogens: PRO, NORET, NORG	Estrogens: E3, E3–16 G, E2–3 S, E2–17G: 0.3–7.3 ng L <sup>-1</sup> Progestogens: PRO: 0.5–47 ng L <sup>-1</sup>	[35]
Saitama (Japan)	WWTP influent, effluent and river water	Androgens: ADD, TES, NAD, AND, EPA, METES, NAN Progestogens: PRO, 17-HPT, 21-HPT, MHPT, DPO, NORET, NORG, MPA, MGA	Influent samples Androgens: EPA, AND: 600–1400 ng L <sup>-1</sup> ADD, TES: 7.9–140 ng L <sup>-1</sup> Progestogens: 21-HPT, DPO, MPA, PRO: 2–10 ng L <sup>-1</sup> Effluent samples Androgens: AND: 5 ng L <sup>-1</sup> Progestogens: 21-HPT, DPO, MPA, PRO: 0.03–1.4 ng L <sup>-1</sup> River samples Androgens: NAD, AND: 0.2–0.5 ng L <sup>-1</sup> Progestogens: DPO, PRO: 0.06–0.15 ng L <sup>-1</sup>	[36]
Rhône-Alpes (France)	Superficial and ground water	Estrogens: αE2, E2, E1, E3, EE Androgens: TES, EPITES, ADD, ETIO, DHT, 5-A-3,17-DIOL, AND, EPA Progestogens: PRO, DROS, NORG, NORET, MEPRO, MEG	Superficial water samples Estrogens: E1: 0.3 ng L <sup>-1</sup> Androgens: TES, ADD: 1.6–3.4 ng · L <sup>-1</sup> Progestogens: NORET, NORG, PRO: 1.7–7 ng L <sup>-1</sup> Groundwater samples Estrogens: E1, E2, αE2, EE: 0.3–3.5 ng L <sup>-1</sup> Androgens: TES, ADD: 1.6–6 ng L <sup>-1</sup> Progestogens: NORET, NORG, PRO: 2.8–11 ng L <sup>-1</sup>	[37]
Gran Canaria (Spain)	WWTP influent and effluent water	Estrogens: E1, E2, E3, EE, DES Androgens: TES Progestogens: MGA, NORG	Influent samples Estrogens: E1, E2, E3, DES: 30–290 ng L <sup>-1</sup> Androgens: TES: 5–40 ng L <sup>-1</sup> Effluent samples Estrogens: DES: 25–190 ng L <sup>-1</sup> Androgens: TES: 1–5 ng L <sup>-1</sup> Progestogens: NOR: 5 ng L <sup>-1</sup>	[43]
Guangzhou (China)	Wastewater sludge	Estrogens: E1, E2, EE, DES Androgens: BLDN, TBL, ADD, NAN, BOL, TES, EPA, METES, 4-OHA, DHT, AND Progestogens: NORET, ETES, NORG, MEPRO, PRO Glucocorticoids: PRD, CORT, CORS, PRDNL, DEXA	Estrogens: E1, E2: 1.6–10.2 ng · g <sup>-1</sup> Androgens: ADD, AND, TBL, TES: 3.2–37.8 ng g <sup>-1</sup> EPA: 206–372 ng g <sup>-1</sup> Progestogens: PRO: 6.0–24.6 ng g <sup>-1</sup>	[48]
Danube River (Hungary)	River water	Androgens: BOL, EPITES, METES, NORTES Progestogens: PRO Glucorticoids: TRIAM, CORS, DEXA, FLUM, PRNL, TRIAM-ACE	Androgens: BOL, EPITES, METES, NORTES: 0.1–0.9 ng L <sup>-1</sup> Progestogens: PRO: 0.2–0.4 ng L <sup>-1</sup> Glucocorticoids: TRIAM, CORS, DEXA, FLUM, PRNL, TRIAM-ACE: 0.01–2.7 ng L <sup>-1</sup>	[51]
Sta. Catarina (Brazil)	Mangrove sediment	Estrogens: E1, E2, E3, EE	Estrogens: E1, E2: 0.7–49.2 ng g <sup>-1</sup> EE: 2.0–129.8 ng g <sup>-1</sup>	[52]
Tarragona (Spain)	Wastewater sludge	Glucocorticoids: BMS, CORS, CORT, DEXA, FMS, MPNL, PRNL, PRD, TRIAM-ACE	Glucocorticoids: CORS: 5.2–6.1 ng g <sup>-1</sup> PRNL: <LOQ-6 ng · g <sup>-1</sup> CORT: nd-<LOQ	[53]
Languedoc-Rusillon (France)	Wastewater sludge	Estrogens: E1, E2, E3, EE	Estrogens: E1, E2, EE: 1–18 ng g <sup>-1</sup>	[54]
Spain	Fish	Estrogens: E1, E2, E3, EE, E1–3S Progestogens: PRO, NORG	Estrogens: E1: <MDL-1.99 ng g <sup>-1</sup>	[55]
Gran Canaria (Spain)	Wastewater sludge	Estrogens: E2, E3, EE	Estrogens: E2: 2.2–100.4 ng · g <sup>-1</sup> E3, EE: 0.9–48.1 ng g <sup>-1</sup>	[56]
Oslo (Norway)	Wastewater sludge	Estrogens: E1, E2, E3, EE	Estrogens: E1	[57]
Taiwan	Sediment	Estrogens: E1, E2, E3, EE	Estrogens: E1: 1.50 ng g <sup>-1</sup>	[60]
Taipei City (Taiwan)	Fish, clam	Estrogens: E1, E2, E3, EE	Estrogens: E1, E2, E3: 0.5–1.4 ng g <sup>-1</sup>	[60]
China	Shrimp, squid and fish	Estrogens: E3, αE2, E1 Androgens: NAN, TES Progestogens: 17-HPT, MEPRO, PRO Glucocorticoids: COR	Estrogens: αE2: 0.4 ng g <sup>-1</sup> Androgens: TES, NAN: 0.3–0.5 ng g <sup>-1</sup> Progestogens: 17-HPT: 0.3 ng g <sup>-1</sup> Glucocorticoids: COR: 0.6 ng g <sup>-1</sup>	[62]
Venice (Italy)	Mussel	Estrogens: E1, E2, E3, EE, MES, DES	Estrogens: EE: 3–38 ng g <sup>-1</sup>	[64]

from conjugates, in contrast to other investigators who only determine the free steroids of biota samples. Prior to extraction, samples are homogenized and, in most cases, crushed and sieved [54,58–62,64,70].

### 3.2.2. Extraction process

Varying techniques are used for the extraction of steroids from solid and biota samples, ranging from traditional liquid-liquid extraction (LLE) [67] to quick easy cheap effective rugged safe (QuEChERS) to matrix solid phase dispersion (MSPD) extraction methods [61,66]. Ultrasonic assisted extraction (UAE), usually used in the extraction of steroids from solid samples [54,58,63,69,70], requires a few minutes from 1 to 2 h. In most cases, 10–50 mL of pure or mixed methanol, ethyl acetate, dichloromethane, acetonitrile, hexane and acetone were used as extraction solvents. Several authors extracted two or three times to ensure maximal isolation of analytes from the matrix [54,58,70]. Others coupled a purifying step to UAE to clean the extract obtained. Xu et al. [69] combined UAE with traditional LLE, while Thomas et al. and Liu et al. [54,63] centrifuged their extracts. Microwave assisted extraction (MAE) and dynamic microwave assisted extraction (DMAE) were also used with solid and biota samples [62,65,68]. The typical 5 mL portions of methanol or acetonitrile used for these extractions are smaller than those used for UAE, and the extraction times for MAE and DMAE are also shorter. Pressurized liquid extraction (PLE), also known as accelerate solvent extraction (ASE), has been developed in the last decade and is as routine as UAE or MAE. Some authors used this technique to extract steroid hormones from sludge and soil samples [59,60,64]. The solvents used in PLE are typically methanol and acetone in varying proportions and some authors (Herrero et al. and Gineys et al. [59,64]) used diatomaceous earth as a dispersive agent. Novel extraction techniques, such as MSPD and QuEChERS, have been recently used for the extraction of estrogens and progestogens from sediments and fish and clam tissues [61,66]. These techniques present the advantage that they can be applied to small amounts of sample (less than 1 g) and use low solvent volumes.

Owing to the complexity of solid and biota samples, a clean-up step is frequently used to eliminate impurities. Solid phase extraction is a common clean-up step for extracts. Some authors dilute, concentrate or dry the extracts and reconstitute them with water before the SPE clean-up. A variety of sorbents are used in SPE clean-up, for example magnesium silicate [70], polymeric [59,60], octadecyl [62,69], alumina [66] or ion-exchange sorbents [63].

The recoveries obtained with these techniques are satisfactory in most cases. For UAE, the recoveries in sludge samples ranged from 62 to 122% for androgens, from 66 to 116% for glucocorticoids [54] and from 44 to 82% for mussels and fish tissue [69,70]. Using MAE and DMAE, the recoveries are similar, over 50% in the extraction of sediments, sludge and aquatic organism tissues [62,65,68], and using PLE, the recoveries are over 45% for estrogens, androgens and progestogens in sludge and soils [60,64]. Novel extraction techniques provide good recoveries for the analysis of estrogens and progestogens in sediments and aquatic organism tissues [61,66].

Table 2 provides references for procedures using USE, MAE and PLE extraction of steroid hormones from solid and aquatic biota samples.

## 4. Determination and quantification

### 4.1. Liquid chromatography

Steroid hormones have traditionally been determined using gas chromatography (GC) [71–74] despite their low volatility and the necessity for derivatization. Moreover, GC analysis is time-

consuming and suffers from low recoveries and the inability to determine compounds such as estriol and the decomposition of ethynyl-estradiol to estrone is not detected. [71,75]. For these reasons, in recent years, liquid chromatography (LC) coupled to optical or mass spectrometer detectors (LC-MS; LC-MS/MS) has been gaining favor over GC. According to Croley et al. [76], chromatographic techniques used to determine hormonal compounds could be ordered by their sensitivity as follows: LC-MS/MS > GC-MS/MS > LC-MS. Because hormone families share similar structures, it is essential to achieve a good chromatographic separation whether a method is performed using an optical detector, or with MS and MS/MS. This is because the hormones within a family have similar molecular weights, and at times, the ionization of distinct compounds produces two unique compounds with the same molecular weight [56].

Reversed phase columns are used by most authors for the analysis of all families of hormones in environmental waters. Octadecyl carbon chain (C<sub>18</sub>)-bonded silica is most frequently used to achieve the separation of steroid hormones [14,23,38,40–55]. To a lesser extent, phenyl-bonded silica columns have been used for estrogen separation in river and tap water [49,56] and alkyl-amide columns have been used to achieve the separation of glucocorticoids, androgens and progesterone in river water [57]. Bridge ethyl hybrid (BEH) C<sub>18</sub> columns are used with ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The solvents used with these columns are in all cases water and organic solvents. Acetonitrile (ACN) [14,23,38,40,42–56] is generally used in the analysis of oestrogens, while methanol (MeOH) is widely used to achieve the separation of androgens, glucocorticoids and progestogens and, sometimes, estrogens [39,41,46,47,53–55,57]. Commonly, the chromatographic separation is performed in gradient mode. However, Lucci et al. [56] reports using the isocratic mode (51:44:5; water:ACN:MeOH) to achieve the separation of six estrogens.

The pH of mobile phase solvents has an important effect on the retention of analytes, and the selectivity of certain analytes can change with pH; therefore, many authors add modifiers to their mobile phase, which is typically water. Water acidified with formic acid [39–41,43,53,55] and acetic acid [14,44,45,55] is used to separate all families of hormonal compounds. Water or methanol with an acidic buffer of acetic acid and ammonium acetate are also used [46,57], while a basic buffer with ammonium acetate and ammonia is rarely used [47].

For the analysis of solid and aquatic biota samples, most authors use an octadecyl carbon chain (C<sub>18</sub>)-bonded silica column to achieve the separation of estrogens, androgens progestogens and corticosteroids [54,59–69]. Other authors, as Pojana et al. [70] use C<sub>8</sub> columns to realize the chromatographic separation of estrogens in mussels. As for the analysis of water samples, bridge ethyl hybrid C<sub>18</sub> columns, are used with UHPLC-MS/MS [61,66]. The organic solvents employed are methanol [59,61,62,65,66] and acetonitrile [54,58–60,63,64,67–70], particularly for the separation of estrogens. The aqueous solvent is water, in some cases modified with formic acid [59,65], acetic acid [62], ammonia [61] or ammonium hydroxide [63]. To obtain a good chromatographic separation, gradient mode is used by most authors [54,59–70], although Froehner et al. and Bhattacharyya et al. [58,67] employed the isocratic mode.

### 4.2. Detectors coupled to liquid chromatography

#### 4.2.1. Optical detectors

Optical detectors have been used in analysis of various hormonal compounds despite their lower sensitivity relative to spectrometric detectors. However, optical methods, such as

ultraviolet-visible (UV) or diode array detection (DAD) have been used to optimize new extraction techniques, such as DLLME-SFO [49], CPE [50] or SBSE [51], for estrogens and progestogens from environmental waters and for traditional extraction techniques, such as SPE [14,38]. The wavelengths used ranged from 197 to 280 nm for estrogens and progestogens; 240 nm is employed most often. With the optical detectors, the limits of detection of the microextraction methods are usually one or two orders of magnitude higher than those methods that employ SPE and mass spectrometry detectors. These limits ranged from 200 to 3000 ng L<sup>-1</sup>, so these combination of methods are not appropriate for environmental analysis of this kind of compounds.

In the analysis of progestogens and androgens in ovarian follicles of perch, the use of an ultraviolet-visible detector at 254 nm has been reported [67]. Finally, Froehner et al. determined the presence of some estrogens in mangrove sediment samples using a UV detector at 274 nm [58], with good reproducibility at concentrations that ranged from 0.7 to 130 ng g<sup>-1</sup>. This range of concentrations is the same that reported other authors that use mass spectrometry detectors.

Other authors have reported the use of fluorescence detectors for the analysis of oestrogens in wastewater samples [77,78] due to the fluorescent properties of 17 $\beta$ -estradiol, estrone and 17 $\alpha$ -ethynylestradiol. Use of fluorescence detectors is advantageous as the limits of detection are comparable to the limits observed with mass spectrometry detectors. The limits of detections obtained with fluorescence detectors are lower than the limits reached with UV detection, ranging from 0.5 to 7.6 ng L<sup>-1</sup>, so they are similar than the limits using mass spectrometry detectors. However, only few estrogens present fluorescence and therefore, the analysis of a wide variety of steroid hormones by fluorescence is not possible.

#### 4.2.2. Mass spectrometry detectors

Mass spectrometry coupled to liquid chromatography is the most powerful analytical technique for the determination of steroid hormones in environmental samples. The interfaces used are mainly electrospray ionization (ESI) in positive and negative modes and atmospheric pressure chemical ionization (APCI) in both modes.

According to López de Alda and Barceló [79], ESI interfaces provide better sensitivities for the determination of oestrogens and progestogens in environmental waters samples. For this reason many authors use these ionization modes for the analysis of all types of steroids in this matrix [23,40–55]. Androgens and progestogens are quantified in positive mode, using the molecular ion (M+H)<sup>+</sup> as a precursor ion. Glucocorticoids are quantified in both modes; the molecular ion is employed in positive mode [54], and formate adducts are used as parent ions in negative mode [53]. For the quantification of estrogens, most authors used the negative mode of ESI [23,40,44–47,52,54], but Chang et al. and Viglino et al. [55,39] quantify estrogens in the positive mode using the dehydrated molecular ion as the precursor ion. Other interfaces, such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), are also typically used in positive mode [39,43,56,57] with molecular ions or dehydrated molecular ions as precursor ions. However, in few cases, negative mode is used [57]. In environmental waters, the limits of detection reached using mass spectrometry detectors are very low. Using APCI and APPI interfaces, the limits are significantly higher (between 3 and 50 ng L<sup>-1</sup>) [39,56] than for ESI interfaces, which reach the limits of detection in the range of hundredth to tenth of ng L<sup>-1</sup> [40–42,46,47,53–55].

For solid and aquatic biota samples, most authors use electrospray ionization coupled to tandem mass spectrometry. As in water analysis, estrogens are measured in negative mode, using the negative molecular ion (M-H)<sup>-</sup> as a parent ion [54,60–63,66,68–70]. Additionally, some authors use this mode for the quantification of glucocorticoids [59,68]. For androgens and progestogens, ESI in positive mode is used in solid and biota samples [54,61,64,68,69]. There are fewer articles reporting the use of APCI and APPI interfaces for the determination of steroid hormones in solid and biota samples because these matrices are less studied than environmental waters. Nevertheless, Snow et al. [65] determined 16 steroid hormones (estrogens, androgens and progestogens) in sediments and soils using an APPI interface in positive mode for all of the compounds. The limits of detection for sediments and soils are similar in all of the studies and range from 0.1 ng g<sup>-1</sup> for androgens and progestogens [64,65] to 3 or 5 ng g<sup>-1</sup> for some estrogens and glucocorticoids [59,60]. On the other hand, the limits of detection in aquatic biota analysis using mass spectrometry detection are similar, lower than 1 ng g<sup>-1</sup> in most cases, for all of the steroid hormones families [61,66,68–70].

Matrix effect is a common problem in the analysis of complex matrices using ESI interfaces, which can lead a signal suppression or enhancement. In clean samples as surface or ground waters the matrix effect is lower than in more complex samples as effluent or influent wastewater or biota samples. Several authors have reported the matrix effect of their analysis and it varies largely. Chang et al. and Liu et al. [41,54] reported no suppression in the analysis of androgens and progestogens in environmental waters, while Vega-Morales et al. [47] reported a small enhancement of the signal between 5 and 15%. Also, Liu et al. [54] calculated a moderate enhancement of the signal for several glucocorticoids. For estrogens most authors determined signal suppression that ranged from 5–15% [39,47,52] to less than 50% [54,66]. For sludge samples Liu et al. determined signal suppressions that ranged from 0 to 40% for all the families of hormonal compounds.

The papers reviewed determined a wide range of concentrations of steroid hormones in environmental waters using these modes of detection. The higher concentrations were detected in influent samples of WWTPs at concentrations of ng L<sup>-1</sup> and even  $\mu$ g L<sup>-1</sup> for compounds such as androsterone [41], ethynylestradiol [44] or cortisone and cortisol [53]. In effluent wastewater treatment plant samples, the concentrations were lower because of the partial removal of steroid compounds by the WWTPs [39,41,47]. In surface, underground and river waters, the concentrations are lower, in most cases below 10 ng L<sup>-1</sup> for compounds such as norethisterone, progesterone, estrone, cortisol, cortisone, testosterone and androstenedione [39–43,53,55,57].

Several steroid hormones have been detected in solid and biota samples too. The highest concentrations detected in solid samples have been in wastewater treatment plant sludge. Muller et al. and Vega-Morales et al. [60,62] reported the presence of estrogens as estrone, estriol, 17 $\beta$ -estradiol and 17 $\alpha$ -ethynylestradiol in concentrations from a few ng g<sup>-1</sup> to 100 ng g<sup>-1</sup> in some sludge samples. Herrero et al. [59] also detected three glucocorticoids, but in lower concentrations. In sediments the concentrations of steroid hormones are lower. Froehner et al. [58] detected estrone, 17 $\beta$ -estradiol and 17 $\alpha$ -ethynylestradiol in mangrove sediments at concentrations ranging from 0.90 to 49 ng g<sup>-1</sup>, while Chen et al. [66] detected estrone at concentrations up to 1.50 ng g<sup>-1</sup> in river sediments. Finally, in many aquatic organisms, low concentrations of steroid hormones have been detected. In yellow croaker, testosterone has been detected at concentrations up to 0.45 ng g<sup>-1</sup>; in shrimp, 17 $\beta$ -estradiol and nandrolone have been detected at concentrations between 0.30 and 0.39 ng g<sup>-1</sup>; and in hairtail, the concentrations of cortisone, 17 $\beta$ -estradiol and 17 $\alpha$ -hydroxyprogesterone range from 0.26 to 0.57 ng g<sup>-1</sup> [68]. Jakimska et al. [61]

detected concentrations up to 1.99 ng g<sup>-1</sup> of estrone in carp, and Chen et al. [66] detected estrone, 17β-estradiol and estril at concentrations up to 1.40 ng g<sup>-1</sup> in fish.

Table 3 shows the concentrations found of hormones in various liquid, solid and biota samples.

## 5. Conclusions

Steroid hormones are present in environmental waters, solids and biota at trace levels, normally in the range of ng L<sup>-1</sup>–ng g<sup>-1</sup>. In the last decades, many extraction and preconcentration techniques have been developed to extract these small amounts of hormones from complex environmental matrices. The application of liquid chromatography with mass spectrometry (LC–MS) has allowed the determination of a large number of compounds. Advantages of combination of extraction and preconcentration techniques to liquid chromatography are: lower quantities of organic solvents; fewer additional steps, such as the derivatization necessary in gas chromatography; and shorter analysis times. These advanced procedures have demonstrated utility in the quantification of trace levels of these compounds with high precision and sensitivity.

Future trends in the analysis of steroids in aquatic environmental samples will be the development of on-line extraction techniques, such as on-line SPE or SPME, to minimize sample handling; the miniaturization of these techniques, allowing for lower quantities of samples via the QuEChERS method or MSPD; and the development of more sensitive mass spectrometry detectors. These more sensitive techniques combined with introduction of biosensors or the use of some species as bioindicators will permit a continuous and exhaustive monitoring of steroid hormones in the aquatic environment.

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### **1.5. Evaluación de riesgo ambiental asociado a hormonas esteroideas**

Como se ha podido comprobar en las secciones anteriores de la presente Tesis Doctoral, las hormonas esteroideas tienen un amplio uso en la actualidad y su entrada al medioambiente es prácticamente continua, por lo que se hace necesario el desarrollo de metodologías que determinen el posible riesgo ambiental asociado a ellas. Las metodologías de gestión de riesgos no se circunscriben al ámbito ambiental, sino que las diferentes administraciones públicas desarrollan directrices en todos los ámbitos en los que una actividad pueda provocar un riesgo, como en finanzas, seguridad, industria, salud pública o vigilancia farmacéutica, siendo este último el ámbito en el que se centre la presente Tesis Doctoral. La Agencia de Alimentos y Medicamentos norteamericana (FDA, *Food and Drug Administration*), por ejemplo, establece que la evaluación de riesgos es indispensable para el control de calidad de un proceso, ya que sin ella no es posible conocer el riesgo de una determinada actividad, ni tomar las medidas necesarias para su control y minimización. En la siguiente figura, extraída de la Guía para la Industria de Gestión de Riesgos se muestran los diferentes pasos de la gestión de riesgos, y cómo la evaluación de riesgos (*risk assessment*) es el primer paso de este proceso [276]:



*concentration*) en el caso de que no se conozca la concentración real del ecosistema afectado.

- Evaluación de los efectos producidos: Cálculo de la concentración prevista sin efecto (PNEC, *predicted no-effect concentration*), la cual será diferente dependiendo del grupo de organismos representativos del medio.
- Cuantificación del riesgo: Cálculo de la relación entre PEC y PNEC. Siempre que PEC/PNEC sea superior a 1, se considerará que existe un riesgo.

En el caso específico de los fármacos y tras el interés y preocupación de la comunidad científica acerca de los efectos tóxicos de éstos, que comenzó a principio de los años 90, las diferentes administraciones públicas con competencias en el ámbito del medio ambiente comenzaron a diseñar regulaciones que predijeran el efecto de los fármacos una vez llegados a un determinado ecosistema. Así, en 1998 la FDA y en 2006 la Agencia Europea de Medicamentos (EMA, *European Medicines Agency*) publicaron sendas guías de evaluación de riesgos para fármacos, las cuales eran necesarias en la comercialización de nuevos fármacos y sirvieron como marco legislativo para los fármacos ya comercializados hasta el momento y cuyos efectos en el medio ambiente comenzaban a ser notorios [278–280]. En la actualidad, la guía desarrollada por la EMA se encuentra en fase de revisión, para que ésta tenga en cuenta la información científica obtenida en la última década y adecue los procedimientos a la situación actual [281]. Como se puede comprobar en la siguiente tabla, ambas guías presentan muchas similitudes y llevan a cabo la evaluación de impacto ambiental de un fármaco de manera muy parecida:

## Capítulo 1. Introducción

Tabla 1.7. Comparación de protocolos diseñados por la FDA y la EMA en la evaluación de riesgos ambientales producidos por compuestos farmacéuticos

Fase de la evaluación de riesgos	Directrices de la FDA (1998) [279]	Directrices de la EMA (2006) [280]
<b>Fase I</b>	<p>Estimación de la exposición</p> <ul style="list-style-type: none"> <li>• Cálculo de la EIC</li> </ul>	<p>Estimación de la exposición</p> <ul style="list-style-type: none"> <li>• Revisión de la persistencia, bioacumulación y toxicidad</li> <li>• Cálculo de la PEC</li> </ul>
<b>Fase II Grado I</b>	<p>Análisis de los efectos producidos (si <math>\frac{LC_{50} \text{ o } EC_{50}}{MEEC} &gt; 1000</math>)</p> <ul style="list-style-type: none"> <li>• Estudio en ecotoxicidad aguda en al menos un organismo representativo del medio</li> </ul>	<p>Análisis inicial del destino ambiental y efectos</p> <ul style="list-style-type: none"> <li>• Estudio de las propiedades físico-químicas y destino</li> <li>• Estudio de los efectos en organismos acuáticos</li> <li>• Cálculo de la PNEC</li> <li>• Evaluación de las aguas subterráneas</li> </ul>
<b>Fase II Grado II</b>	<p>Análisis de los efectos producidos (si <math>1000 &gt; \frac{LC_{50} \text{ o } EC_{50}}{MEEC} &gt; 100</math>)</p> <ul style="list-style-type: none"> <li>• Estudio en ecotoxicidad aguda en un grupo de organismos acuáticos (pez, invertebrado y alga) o terrestres (planta, gusano de tierra y microorganismos presentes en el suelo)</li> </ul>	<p>Análisis extenso del destino ambiental y efectos</p> <ul style="list-style-type: none"> <li>• Re-cálculo y ajuste de la PEC</li> <li>• Análisis extenso de los efectos producidos</li> <li>• Estudio del transporte en tierra y efectos</li> </ul>
<b>Fase II Grado III</b>	<p>Análisis de los efectos producidos (si <math>100 &gt; \frac{LC_{50} \text{ o } EC_{50}}{MEEC} &gt; 10</math>)</p> <ul style="list-style-type: none"> <li>• Estudio en ecotoxicidad crónica para organismos terrestres o acuáticos</li> </ul>	

En el caso de las directrices seguidas por la EMA, la PEC en aguas superficiales se calculará como:

$$PEC_{surface\ water} (\mu g \cdot L^{-1}) = \frac{DOSE_{ai} \cdot F_{pen}}{WASTEW_{inhab} \cdot DILUTION \cdot 100} \quad (3)$$

Donde:

- PEC es la concentración ambiental prevista
- DOSE es la dosis máxima de ingrediente activo consumida por habitante ( $mg \cdot habit^{-1} \cdot dia^{-1}$ )
- $F_{pen}$  es la penetración de la droga en el mercado (%)
- $WASTEW_{inhab}$  es el volumen de agua residual producida por habitante y día ( $L \cdot habit^{-1} \cdot dia^{-1}$ ). Se puede considerar igual a 200 por defecto
- DILUTION es el factor de dilución que sufren las aguas residuales al llegar al medio. Se puede considerar por defecto igual a 10
- 100 es un factor de corrección de los porcentajes utilizados.

En el caso de que exista un riesgo para el medioambiente, la PEC deberá ser revisada y refinada, teniendo en cuenta factores como la fracción directamente emitida a las aguas superficiales, el factor de adsorción sobre materia orgánica o la concentración del fármaco existente de manera previa en el medio receptor.

Por su parte, la FDA recomienda utilizar la siguiente expresión para realizar la primera estimación de la concentración en el medio (*EIC, Expected Introductory Concentration*)

$$EIC(\mu g \cdot L^{-1}) = A \cdot B \cdot C \cdot D \quad (4)$$

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Donde:

- A es la cantidad de compuesto activo producido para su uso directo ( $\text{kg}\cdot\text{año}^{-1}$ )
- B es el cociente 1/litros por día de aguas residuales tratadas en el sistema público
- C es el número de años dividido entre 365 días
- D es igual a  $10^9$ , se utiliza como factor de conversión entre kg y  $\mu\text{g}$ .

Esta concentración se considerará como concentración máxima esperada en el medio (MEEC), a menos que la concentración detectada en el medio sea mayor, en cuyo caso se usará como MEEC. Seguidamente se utilizará la dosis letal media ( $LC_{50}$ ) o dosis con efectos media ( $EC_{50}$ ) para realizar el siguiente cociente:

$$\frac{LC_{50} \text{ o } EC_{50}}{MEEC} \quad (5)$$

Dependiendo de si el valor de este cociente es superior a 1000, 100 o 10 se pasará a la fase II grados I, II o III, respectivamente.

Una vez calculadas estas concentraciones estimadas, se procederá a enfrentar el dato con la PNEC obtenida para las diferentes especies presentes en el medio estudiado. Como se indicó arriba, si el cociente entre PEC (o EIC) y PNEC es superior a 1, se considerará que existe un riesgo medioambiental, por lo que habrá que proceder a realizar acciones de remediación para evitar que este se produzca, o para minimizar los posibles efectos si la contaminación ya se ha producido.

La evaluación de riesgos es una herramienta muy poderosa utilizada por diversos autores en estudios de campo de presencia de hormonas en el medio acuático, ya que sirve para indicar las zonas de un sistema medioambiental, como por ejemplo una cuenca hidrográfica donde se produzcan vertidos de aguas residuales, donde sean necesarias medidas para minimizar el impacto negativo. En este sentido, Hernando y colaboradores, realizaron el análisis del riesgo ambiental ligado a diferentes fármacos, entre ellos, varias hormonas esteroideas como el  $17\alpha$ -etinilestradiol o el dietilestilbestrol [282]. La evaluación fue realizada tomando datos de concentraciones reales en aguas residuales, superficiales y sedimentos, y, tras enfrentar los valores obtenidos de la bibliografía con las PNEC de cada compuesto, estimaron que el riesgo medioambiental ligado a hormonas en aguas residuales era bajo, no siendo evaluado en aguas superficiales y sedimentos [282]. Por otra parte, Stuer-Lauridsen y colaboradores obtuvieron resultados contradictorios al evaluar el riesgo ambiental ligado a combinaciones de gestodeno y estrógenos y desogestrel y estrógenos [283]. Dependiendo del organismo utilizado para hacer el test de concentración prevista sin efecto, se podía asegurar si había riesgo o no. Para la especie estandarizada (*Daphnia magna*), el cociente PEC/PNEC quedó muy por debajo de 1, mientras que para otra especie no utilizada de manera habitual en los test de toxicidad (*Medicago sativa*), se obtuvo un índice de riesgo ambiental más alto, aunque inferior a 1 [283]. Este estudio muestra, por tanto, la importancia de la elección de la especie representativa del ecosistema estudiado.

Por otra parte, en el caso particular de los estrógenos, en algunos estudios se ha calculado la actividad disruptora endocrina, también

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denominada estrogénicidad, que puede ser considerada una evaluación análoga y complementaria al riesgo ambiental. Para evaluar la estrogénicidad, es necesario hacer una normalización de la capacidad estrogénica de las diferentes especies disruptoras, pues cada uno de ellas tiene una capacidad diferente, para lo cual se calcula la concentración de equivalentes de estradiol (EEQ). La razón de que se utilice el estradiol como compuesto de referencia, es que se conocen bien sus efectos en el medio y, además, es uno de los pocos compuestos disruptores endocrinos que cuentan con regulación en el ámbito de las aguas superficiales. La EEQ se puede definir como la concentración del contaminante ( $C_i$ ) por el factor de equivalencia con respecto al estradiol ( $EEF_i$ ):

$$EEQ_i = C_i \cdot EEF_i \quad (6)$$

Por su parte, el factor de equivalencia con respecto al estradiol puede calcularse como:

$$EEF_i = \frac{EC_{50E2}}{EC_{50i}} \quad (7)$$

Donde  $EC_{50,E2}$  es la concentración media de estradiol que produce efectos en organismos y  $EC_{50,i}$  es la concentración media que produce efectos del compuesto estudiado. Para conocer las concentraciones medias con efectos tanto del E2, como del compuesto en estudio se pueden llevar a cabo diversos ensayos biológicos, los cuales pueden ser *in vivo* o *in vitro* [284]. De estos últimos se pueden destacar aquellos que miden la afinidad de una sustancia para unirse a un receptor estrogénico, los que miden la capacidad de una sustancia de estimular el crecimiento celular asociado a un estrógeno o los que miden la capacidad de una sustancia de activar procesos de transcripción genética [284].

Una vez conocida la capacidad estrogénica de los compuestos presentes en la muestra, se puede calcular la estrogenicidad de ésta, ya que se ha demostrado que la capacidad estrogénica de un muestra es la suma de las capacidades disruptoras de las especies en disolución [285]:

$$EEQ_t = \sum EEQ_i \quad (8)$$

El estudio de la capacidad estrogénica de las aguas ha sido realizado por diversos autores, como por ejemplo Brix y colaboradores [286]. En este estudio determinaron la concentración de 19 compuestos alquilfenólicos y estrógenos en la cuenca del río Llobregat (Cataluña, España) y, posteriormente, calcularon la concentración equivalente total de estradiol de los puntos estudiados, comprobando que, en muchos de ellos, y durante las diferentes campañas realizadas, se superaba la concentración límite de  $1 \text{ ng}\cdot\text{L}^{-1}$  de E2, a partir de la cual pueden observarse efectos estrogénicos en la biota acuática. Debido a la multitud de valores de EEF obtenidos por diferentes estudios para los compuestos estudiados, Brix y colaboradores decidieron hacer la media de los valores de EEF encontrados en la bibliografía para los diferentes compuestos, al igual que realizó Vega-Morales y colaboradores [287]. En este trabajo, también se evaluó la capacidad estrogénica de diferentes efluentes de estaciones depuradoras de aguas residuales, y se comprobó que prácticamente siempre se superaba el umbral de  $1 \text{ ng}\cdot\text{L}^{-1}$  de E2, lo que demuestra la hipótesis de que los efluentes de las EDAR tienen una alta capacidad estrogénica, como han concluido estudios anteriores.

### 1.6. Tratamiento de datos experimentales

En el desarrollo de un buen método analítico es necesario valorar la calidad del método una vez desarrollado, evaluando su precisión, límites de detección y cuantificación, linealidad y exactitud. A continuación, se exponen la forma en la que estos parámetros han sido evaluados en los trabajos descritos en el apartado experimental de la presente Tesis Doctoral.

#### – Precisión

Todo método analítico cuantitativo debe ser preciso a la hora de calcular una determinada concentración y la precisión de éste debe calcularse en condiciones de repetitividad y reproducibilidad. En este sentido, se realiza el estudio *intra-day*, es decir analizando las muestras un mismo día, e *inter-day*, que significa que se hacen réplicas del análisis durante varios días, consecutivos o no, para evaluar la precisión del método desarrollado. Para ello, se calcula la desviación estándar relativa (RSD, *relative standard deviation*) de un conjunto de, como mínimo, 6 muestras aplicando la siguiente fórmula matemática:

$$RSD(\%) = \frac{\sigma}{\bar{x}} \cdot 100 \quad (9)$$

$$\bar{x} = \frac{1}{N} \sum_{i=1}^N x_i \quad (10)$$

$$\sigma = \sqrt{\frac{1}{N-1} \sum_{i=1}^N (x_i - \bar{x})^2} \quad (11)$$

Donde  $\bar{x}$  es la media aritmética de las réplicas,  $\sigma$  es la desviación estándar de éstas y N el número de réplicas.

### – Linealidad

La linealidad de un método analítico se refiere a la proporcionalidad entre la concentración del compuesto estudiado y la señal analítica que éste proporciona. Es necesario que la linealidad se cumpla en el intervalo de concentraciones que se obtienen en las muestras para asegurar la calidad del método. La linealidad se evalúa en las curvas de calibrado construidas para un método, las cuales pueden ser externas, si se realizan disolviendo diferentes cantidades de patrón puro en disolvente, o internas, si se contaminan las muestras a diferentes concentraciones y se les aplica el método analítico completo. En los trabajos presentados en esta Tesis Doctoral se han utilizado ambas modalidades de curva de calibrado, dependiendo del método analítico desarrollado, construyendo siempre las rectas de calibración con, al menos, 6 niveles. Para realizar la regresión lineal de los datos y obtener así la línea de tendencia se ha utilizado la técnica de ajuste de mínimos cuadrados, obteniéndose el coeficiente de determinación ( $r^2$ ), el cual es el coeficiente de Pearson al cuadrado. El coeficiente de Pearson se calcula con la siguiente fórmula:

$$r_{x,y} = \frac{\sigma_{xy}}{\sigma_x \sigma_y} = \frac{n \sum x_i y_i - \sum x_i \sum y_i}{\sqrt{n \sum x_i^2 - (\sum x_i)^2} \sqrt{n \sum y_i^2 - (\sum y_i)^2}} \quad (12)$$

Los valores que puede tomar el coeficiente de determinación ( $r^2$ ) van de +1 a -1, donde +1 significa correlación lineal total positiva, 0 significa que no existe correlación lineal y -1 correlación lineal total

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negativa. En los trabajos de esta Tesis Doctoral se ha determinado que los coeficientes de determinación sean superiores a 0,990 para ser considerados.

### – Límites de detección y cuantificación

La idoneidad de un método analítico está íntimamente ligada a los límites de detección y cuantificación que éste presente, es decir, a las concentraciones mínimas que sea capaz de detectar y cuantificar. Debido a que las hormonas esteroideas se encuentran en el medio a niveles traza y ultratrazas, es necesario desarrollar métodos con límites de detección y cuantificación muy bajos, que se encuentren en el rango de los  $\text{ng}\cdot\text{L}^{-1}$ . De las diferentes metodologías existentes para calcular el límite de detección se ha decidido utilizar aquella que indica que *“El límite de detección viene dado por la concentración de analito que genera una relación señal/ruido (signal/noise) igual a tres”*. La ecuación utilizada ha sido la siguiente y se ha realizado utilizando como mínimo un triplicado de muestras:

$$LOD = \frac{\bar{x}_{\text{señal}}}{\bar{x}_{\text{ruido}}} = 3 \quad (13)$$

En lo que respecta al límite de cuantificación, la definición formal indica que *“El límite de cuantificación viene dado por la concentración de analito que genera una relación señal/ruido (signal/noise) igual a diez”*. Al igual que el límite de detección se utilizó, como mínimo, un triplicado de muestras para calcularlo utilizando la ecuación:

$$LOQ = \frac{\bar{x}_{\text{señal}}}{\bar{x}_{\text{ruido}}} = 10 \quad (14)$$

### – Recuperación

La recuperación es un parámetro de calidad que afecta al método de extracción. Normalmente, los métodos de extracción y preconcentración de muestras no son capaces de extraer el 100% del analito que la muestra contiene, razón por la cual se hace necesario el cálculo de la capacidad de extracción del método para poder calcular las concentraciones ambientales. Sin este parámetro, las concentraciones ambientales calculadas estarían subestimadas. De manera general, en la presente Tesis Doctoral se ha utilizado el procedimiento indicado por Matuszewski y colaboradores [288] en el que la recuperación del método se calcula como la relación entre el extracto de una muestra contaminada a una determinada concentración (C) y el extracto de la muestra sin contaminar, el cual se contamina con los analitos de análisis tras realizar la extracción (B):

$$\text{Recuperación (\%)} = \frac{C}{B} \cdot 100 \quad (15)$$

De esta manera, una recuperación del 0% indica que el método de extracción no es capaz de extraer ninguna cantidad de analito de la muestra, mientras que una recuperación del 100% indica la extracción total del analito presente en la muestra.

### – Efecto matriz

El efecto matriz es un fenómeno que se produce en los análisis cromatográficos que utilizan espectrometría de masas con ionización de electrospray (MS-ESI) como método de determinación. El efecto matriz consiste en una supresión o

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incremento de la señal analítica provocada por las interferencias presentes en el extracto de la muestra analizada, las cuales provocan que la ionización de la molécula o moléculas fruto del análisis sea diferente a la ionización que se produce cuando el analito se encuentra disuelto en un disolvente puro. Este fenómeno ha sido claramente determinado en el análisis de disruptores endocrinos [197], y diversos autores han desarrollado expresiones matemáticas para calcular la supresión (o en su caso, realzamiento) de la señal analítica. Por una parte, Matuszewski y colaboradores lo calculan como el cociente entre la señal analítica producida por el extracto de una muestra contaminado a una determinada concentración (B) frente a la señal producida por el analito disuelto en disolvente puro a esa misma concentración (A) [288]:

$$\text{Efecto Matriz (\%)} = \frac{B}{A} \cdot 100 \quad (16)$$

Utilizando esta expresión, un valor de efecto matriz igual al 100% indica que no existe efecto matriz, mientras que valores inferiores indican que se produce supresión de la señal analítica.

Por otra parte, Vieno y colaboradores han desarrollado otra expresión que mide la supresión iónica que sufre la muestra [289].

En este caso, los autores utilizan la siguiente expresión:

$$\text{Supresión iónica (\%)} = \frac{A_s - (A_{sp} - A_{usp})}{A_s} \cdot 100 \quad (17)$$

Donde  $A_s$  corresponde a la señal analítica de una disolución del compuesto en disolvente puro (*standard*),  $A_{sp}$  es la señal obtenida al analizar un extracto de la muestra analizada, contaminada al mismo nivel que  $A_s$  (*spiked*) y  $A_{usp}$  es la señal obtenida al analizar un extracto de la muestra sin contaminar (*non-spiked*). Los valores

positivos obtenidos indicarán que existe supresión iónica, mientras que los valores negativos indicarán que se está produciendo un incremento de la señal analítica con respecto al estándar puro. Un valor igual a 0 indicará que no existe efecto matriz en la muestra analizada, y por lo tanto que no se produce ni supresión ni incremento de la señal. En la presente Tesis Doctoral se ha utilizado el algoritmo de Vieno y colaboradores para calcular el efecto matriz de las diferentes muestras analizadas, utilizando siempre un mínimo de tres muestras para realizar los cálculos.

Además de valorar la calidad de un método analítico evaluando los parámetros analíticos anteriormente descritos, es necesario estudiar y optimizar todas las variables por las que se puede ver afectado el método de análisis. El diseño experimental se puede realizar estudiando variable a variable, es decir, dando valores a una determinada variable mientras todas las demás se mantienen fijas a un determinado nivel. Sin embargo, esta técnica de optimización presenta un importante inconveniente, ya que, en muchos casos, las variables críticas de un proceso de extracción o determinación están relacionadas entre sí. Por esta razón, en el desarrollo de los métodos de extracción y preconcentración de la presente Tesis Doctoral se han utilizado diseños factoriales de diferentes variables a múltiples niveles con orden aleatorio, para minimizar errores. Los diseños factoriales se han establecido como el método de diseño experimental más extendido debido a que permiten reducir la cantidad de ensayos que se deben realizar y además permite evaluar las correlaciones que existen entre variables, así como la influencia de cada una sobre el método completo [290–292]. Los diseños experimentales aquí descritos se han

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llevado a cabo utilizando el software informático Minitab 15.0 para la creación de los diseños factoriales y éste mismo programa o el programa informático IBM SPSS versión 11.0 para calcular las correlaciones, tanto de cada variable con el método, como entre variables.

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## Capítulo 2. Objetivos

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Tal y como se ha expuesto en la introducción, el desarrollo de la sociedad actual ha provocado que el ser humano sea responsable de la llegada al medio de cantidades importantes de hormonas esteroideas, las cuales suponen un riesgo latente para los ecosistemas receptores. Por esta razón, el principal reto de la química analítica durante las últimas décadas ha sido el desarrollo de metodologías de análisis que permitan la detección y cuantificación de estos contaminantes.

En muchas ocasiones éstos se encuentran a niveles traza o ultratrazo, lo que ha hecho necesario que se desarrollen también metodologías que permitan su extracción y preconcentración de las matrices en las que se encuentran hasta llegar a un nivel en que puedan ser fácilmente medibles por las técnicas de cuantificación. Las tendencias actuales en este campo pasan por el desarrollo de mejoras de las técnicas ya existentes que permitan su miniaturización y automatización para

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hacerlas más sencillas, rápidas, reproducibles, respetuosas con el medio ambiente y asequibles, pero también del desarrollo de nuevas metodologías basadas en estas premisas.

El desarrollo de estas metodologías no es sólo un avance en el campo de la Química Analítica, sino que suponen un importante beneficio también a la sociedad, ya que permiten conocer de manera más precisa los niveles de hormonas esteroideas que existen en los diferentes compartimentos medioambientales en los que vive y de los que se nutre la sociedad. Los resultados obtenidos serán la base para desarrollar reglamentaciones y legislaciones que permitan que el impacto de las hormonas esteroideas en el medio sea cada vez menor.

Teniendo todo esto en cuenta, el objetivo general de la presente Tesis Doctoral es desarrollar y aplicar nuevas metodologías de extracción y determinación de hormonas esteroideas pertenecientes a cuatro de las familias en las que se dividen:

- Estrógenos:  $17\beta$ -estradiol,  $17\alpha$ -etinilestradiol, estriol, estrona y dietilestilbestrol
- Andrógenos: Testosterona, nandrolona y boldenona
- Progestágenos: Progesterona, noretisterona, levonorgestrel y acetato de megestrol
- Glucocorticoides: Cortisona, prednisona y prednisolona

En este ámbito y de forma más concreta, los objetivos específicos perseguidos en esta Tesis Doctoral son los siguientes:

- 1) Establecer las condiciones óptimas de separación, detección y cuantificación de hormonas esteroideas utilizando como

técnica instrumental la cromatografía líquida de ultra resolución acoplada a detectores de fluorescencia y de espectrometría de masas en tándem.

- 2) Desarrollar y optimizar una metodología basada en extracción en fase sólida que permita extraer y preconcentrar hormonas esteroideas de diferentes familias presentes en aguas residuales, tanto de entrada como de salida de estaciones depuradoras de aguas residuales.
- 3) Desarrollar y optimizar una metodología de extracción en fase sólida en línea (on-line) acoplada a un sistema de cromatografía líquida de ultra resolución que permita la extracción, preconcentración, separación y determinación de hormonas esteroideas presentes en aguas residuales de la isla de Gran Canaria.
- 4) Desarrollar un proceso más selectivo de la extracción en fase sólida tradicional utilizando polímeros impresos molecularmente que permita la extracción y preconcentración de estrógenos, tanto libres como conjugados, en muestras de aguas residuales tanto urbanas, como especiales (aguas residuales de una zona veterinaria hospitalaria).
- 5) Comparar la efectividad de la extracción en fase sólida utilizando polímeros impresos molecularmente y la tradicional en lo que respecta a sensibilidad, selectividad y reproducibilidad del método de extracción.
- 6) Desarrollar y optimizar una nueva técnica de extracción miniaturizada denominada extracción por adsorción sobre

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tejido químicamente modificado que permita la extracción eficiente de andrógenos y progestágenos presentes en aguas residuales urbanas, procedentes de una zona hospitalaria, así como en orina.

- 7) Optimizar una metodología basada en extracción asistida por microondas para la extracción de hormonas esteroideas presentes en muestras de lodos de estaciones depuradoras de aguas residuales.
- 8) Estudiar las condiciones óptimas de una metodología basada en extracción asistida por microondas para extraer hasta quince hormonas esteroideas de diferentes tejidos de peces como músculo, piel y vísceras.
- 9) Aplicar las metodologías de extracción, separación y determinación desarrolladas a muestras medioambientales de carácter líquido, sólido y biológico de la isla de Gran Canaria para evaluar la presencia de estrógenos, andrógenos progestágenos y glucocorticoides en éstas.

## Capítulo 3. Parte Experimental y Resultados

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En el siguiente apartado se detallan los resultados obtenidos en los diferentes trabajos llevados a cabo para realizar la presente Tesis Doctoral. En ellos, se han optimizado y desarrollado diferentes métodos analíticos que permiten la extracción, preconcentración, separación y determinación de hasta quince hormonas esteroideas. Estos métodos se han aplicado tanto en matrices líquidas (trabajos 3.1.1, 3.1.2, 3.1.3, 3.1.4 y 3.1.5) como en matrices sólidas y biológicas (trabajos 3.2.1 y 3.2.2).

Los trabajos presentados a continuación siguen las nuevas tendencias en química analítica, enfocadas en el desarrollo de métodos simples y rápidos que permitan la extracción, preconcentración y determinación de gran variedad de compuestos con la adecuada precisión, selectividad y sensibilidad. En este sentido, los trabajos descritos en los apartados 3.1.1 y 3.1.2 se centran en el desarrollo de

### *Capítulo 3. Parte experimental y resultados*

metodologías de extracción basadas en la extracción en fase sólida convencional para la determinación de hormonas esteroideas de diferentes subfamilias.

A continuación, en los trabajos descritos en los apartados 3.1.3 y 3.1.4 se optimizan métodos basados en extracción en fase sólida pero que siguen las tendencias actuales de automatización y desarrollo de nuevos materiales. En el primero de estos trabajos se presenta un método de SPE en modalidad *on-line*, en los que se minimiza el uso de disolventes, así como los tiempos de análisis y la manipulación de las muestras. Por su parte, en el trabajo descrito en el apartado 3.1.4 se desarrolla una metodología de extracción en fase sólida utilizando polímeros impresos molecularmente, específicamente diseñados para la extracción selectiva de estrógenos presentes en muestras líquidas.

Finalmente, el quinto trabajo presentado en la sección 3.1 desarrolla otra de las tendencias actuales de la química analítica como es la miniaturización. En este trabajo se optimiza una metodología basada en la extracción por adsorción sobre tejidos modificados químicamente. Dicha técnica se ha desarrollado recientemente y se presenta como un potencial sustituto de las técnicas tradicionales de extracción debido a su facilidad de uso y excelentes características analíticas.

A continuación, en el apartado 3.2. se desarrollan sendas metodologías basadas en la extracción asistida por microondas para la extracción de hasta 15 hormonas esteroideas presentes en matrices sólidas como lodos de estaciones depuradoras de aguas residuales y muestras de tejido de peces. El análisis de dichas muestras constituye un reto debido a su complejidad y gran cantidad de interferencias, por lo que

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se han estudiado intentando conseguir las mayores selectividades posibles.

En todos los métodos desarrollados se ha utilizado la cromatografía líquida de ultra resolución con dos tipos de detectores: fluorescencia y espectrometría de masas. En ambos casos se ha integrado la extraordinaria capacidad de separación de la cromatografía líquida de ultra resolución con la sensibilidad y selectividad de los detectores de fluorescencia y espectrometría de masas, obteniéndose metodologías de separación y determinación con bajos límites de detección y altas reproducibilidades.





### 3.1. Análisis de muestras líquidas

#### 3.1.1. Optimización de un procedimiento basado en la extracción en fase sólida combinada con cromatografía líquida de ultra resolución con detección fluorescente para determinar estrógenos en aguas depuradas.

Los estrógenos son uno de los grupos de contaminantes emergentes que más han preocupado a la comunidad científica internacional debido a la cantidad de efectos nocivos que pueden tener en los ecosistemas acuáticos a concentraciones de, incluso,  $1 \text{ ng}\cdot\text{L}^{-1}$ . Por ello, se hace absolutamente necesario el desarrollo de metodologías analíticas que permitan la extracción de estos contaminantes, así como su determinación en los puntos principales de entrada al medio ambiente, las estaciones depuradoras de aguas residuales. Los estrógenos como el  $17\beta$ -estradiol, el estriol o el  $17\alpha$ -etinilestradiol presentan la particularidad de que son moléculas fluorescentes, por lo que pueden ser analizadas con precisión y sensibilidad en un equipo de cromatografía líquida con detección de fluorescencia.

En esta comunicación corta se expone, de una manera muy resumida, la optimización de un método cromatográfico para conseguir la separación de los compuestos en estudio, lo cual es un paso vital en cromatografía con detección por métodos ópticos, así como las condiciones de detección por fluorescencia para tres estrógenos, el estriol, el  $17\beta$ -estradiol y el  $17\alpha$ -etinilestradiol; así como el estrógeno conjugado  $17\beta$ -estradiol-glucurónido.

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En primer lugar, se optimizó el método cromatográfico para conseguir la separación de los compuestos en estudio, así como las condiciones de detección por fluorescencia.

Debido a las diferentes características que presentan los compuestos en estudio, es necesario estudiar qué tipo de medio adsorbente es el más adecuado, así como las condiciones experimentales en que debe producirse la extracción en fase sólida.

Para ello, estudiamos tres tipos de cartuchos comerciales de SPE para comprobar en cuál de ellos se producía una mejor adsorción de los analitos estudiados. Además, estudiamos las variables determinantes en la extracción como son el pH, fuerza iónica y volumen de la muestra. Se realizó un diseño factorial con el que se pudo comprobar la influencia de cada variable en la eficacia del método de extracción así como las correlaciones existentes entre variables. Seguidamente, con las variables que presentaban mayor correlación se procedió a construir superficies de respuesta para evaluar las condiciones óptimas de extracción.

El presente trabajo fue publicado en la revista *Luminescence* (2012), 27:560-561 y fue considerado como punto de partida para el desarrollo del trabajo descrito en el apartado 3.1.2 de la presente Tesis Doctoral.

**Acknowledgements**

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**Optimization of a solid-phase extraction procedure combined with ultra-high performance liquid chromatography and fluorescence detection to determine estrogens in wastewater samples**

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**Keywords:** Estrogenic compounds, UHPLC, fluorescence detection, SPE

**Introduction**

In recent years, the ecosystem problems, the increasing of water demand and the continuous discovery of new emerging pollutants have attracted the attention of scientific community. This is the reason why many researches have been focused on developing new methodologies to determine all substances with environmental interest.

Within this broad group of compounds, the use of estrogenic compounds has increased exponentially nowadays. Currently, there are some investigations that determine the presence of estrogenic compounds in environmental water and wastewater samples (1,2).

In this research, a solid phase extraction (SPE) procedure was optimized and coupled to ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FD) for the determination of a group of four estrogens: estriol,  $\beta$ -estradiol,  $17\beta$ -estradiol glucuronide and  $17\alpha$ -ethinyl estradiol. All parameters involved in solid-phase extraction were optimized, such as, type of SPE cartridge, sample volume, pH and ionic strength of sample, desorption volume and wash step. The results were evaluated to obtain optimum extraction conditions.

**Experimental**

**Chromatographic separation**

An UHPLC system with fluorescence detector (excitation and emission wavelength were 280 and 310 nm respectively) from Waters (Madrid, Spain) and a  $C_{18}$  column were used. 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  $0.5 \text{ mL} \cdot \text{min}^{-1}$ .

**Optimization of SPE method**

The optimization was carried out using Milli-Q water spiked with a solution of estrogens to obtain a final concentration of  $250 \mu\text{g} \cdot \text{L}^{-1}$ .

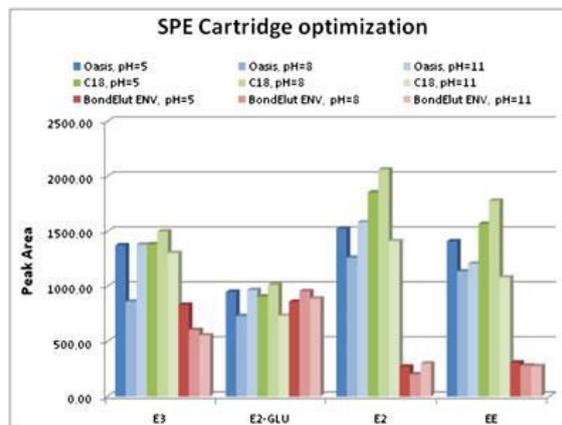


Figure 1. Optimization of SPE cartridges.

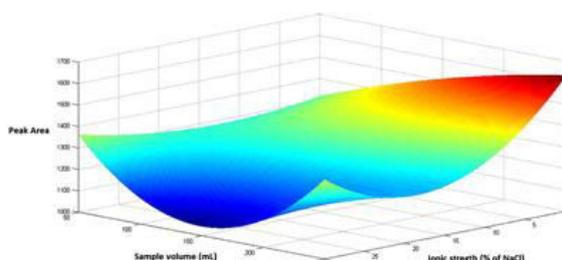


Figure 2. Effect of ionic strength and sample volume on the SPE extraction for Estriol.

The SPE cartridges used were OASIS, SepPak  $C_{18}$  (both from Waters, Madrid, Spain) and BondElut ENV (from Agilent, Madrid, Spain). After choose the optimum cartridge, the parameters studied were pH (3, 8 and 11), ionic strength (0, 15 and 30% of NaCl) and sample volume (50, 100 and 250 mL). The experimental design used for the optimization was obtained using Statgraphics Plus software 5.1 and the statistics study was done with IBM SPSS Statistics 19. 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 studied to complete the optimization of the SPE process.

**Results**

From the results obtained in the study of different cartridges, we can observe that the better signals are found for SepPak  $C_{18}$  cartridge (figure 1).

The pH, ionic strength and sample volume were optimized using a  $2^3$  design, followed by a  $3^2$  design with ionic strength and sample volume because they presented the higher correlations. Figure 2 shows the response surface obtained for the estriol, where can be observe that the best conditions were 0% of NaCl and 250 mL of sample volume.

Finally, the desorption volume and the wash-step were fixed at 2 mL of methanol in one step and 5 mL of Milli-Q water without methanol respectively. In these conditions we achieve a preconcentration factor of 125.

In accordance with the obtained results, the optimum conditions for SPE procedure were: SepPak  $C_{18}$  cartridge, 250 mL

Abstract

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.

**Acknowledgement**

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**Application of the bacterial bioluminescence *vibrio fischeri* method to assessment of EC50 as a marker of toxicity for several pesticides**

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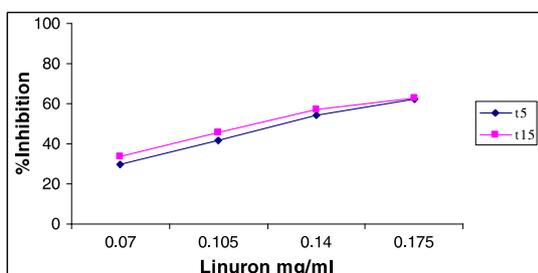
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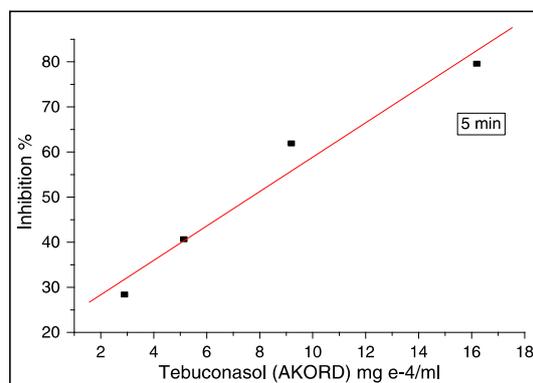
**Keywords:** bioluminescence method; EC50 database; pesticides; toxicity; *Vibrio Fischeri*

**Introduction**

Due to the extensive use of pesticides in agriculture, their presence at the trace level in the environment constitutes a subject of concern for their possible toxic effects on human health. Therefore, in order to correctly estimate the toxicity of pesticides, it is essential for a research laboratory to establish a database of EC50 parameter (i.e. the pesticide efficient concentration yielding a 50 % signal inhibition) concerning the pesticides currently utilized in the country (1). The aim of this work is to determine the EC 50 values of several pesticides used in R. Macedonia by means of the Microtox<sup>®</sup> bioluminescence method, with the marine bacteria *Vibrio Fischeri* as test organism.



**Figure 1.** Inhibition of the *Vibrio fischeri* bioluminescence for the pesticide linuron after exposure times of 5 and 15 min.



**Figure 2.** Inhibition of the *Vibrio fischeri* bioluminescence for the pesticide tebuconazole after exposure time of 5.

**Experimental**

The EC50 database was established for the following pesticides used in the R. Macedonia (2): (a) standard substances: fenoxaprop-p-ethyl, metsulfuron-methyl, mecoprop, linuron, chlorothalonil, cyprodinil, dichlobenil,  $\alpha$ -endosulfane, trifluraline, dimethoate, glyphosate, 2,4-dichlorophenoxyacetic acid (2,4-D), paraquat-dichloride, and (b) commercial products: tebuconazole (Akord), clomazone (Gamit), methomyl (Lannate), difenoconazole (Difcor), thiamethoxam (Actara), malathion (commercial emulsion), kresoxim-methyl (commercial powder). Toxicity measurements were performed with a luminometer LKB 1250, according to the international procedure (DIN/EN/ISO 11348-2), using the *V. Fischeri* luminescent bacteria test and a bacteria activation reagent, LCK 487 LUMISTOX (Hach Lange, Germany) (3, 4). The luminescent bacteria were thawed immediately prior to reactivation. According to the pesticide solubility, sample solutions were either prepared in distilled water or in a water-acetone-ethanol 96/3/1 v:v:v mixture. The pH of the samples was  $7.0 \pm 0.1$ . In all cases, the bioluminescence measurements were performed after 5 and 15 min of exposure of the *V. Fischeri* bacteria to the pesticide solutions at 15 °C (5).

**Results and discussion**

For all pesticides under study, linear standard curves of the percentage of bioluminescence intensity inhibition vs. the pesticide concentration were obtained. Curve analysis allowed us to calculate the EC50 parameter values for the various examined pesticides. Fig. 1 and 2 show two examples of these curves, in the case of linuron, a standard pesticide and tebuconazole, a commercial pesticide.

Our results demonstrated that the pesticide toxicity values very widely varied according to the compound. In the case of the standard substances EC50 values ranged from 0.00069 and 0.0009 mg/mL for dimethoate to 0.83 and 0.66 mg/mL for trifluraline, respectively, for 5 and 15 min exposure, whereas, in the case of commercial products, the corresponding EC50 values were comprised, respectively, between 0.00026 and 0.00028 mg/mL for difenoconazole and between 0.744 and 0.577 mg/mL for methomyl.

Therefore, since the EC50 parameters vary inversely with toxicity, we found that the toxicity order was as followed for the standard pesticides under study: dimethoate > fenoxaprop-p-ethyl > metsulfuron > 2, 4-D > linuron > mecoprop > dichlobenil > glyphosate > paraquat-dichloride > cyprodinil > chlorothalonil >  $\alpha$ -endosulfan > trifluraline. In the case of the commercial pesticides,

#### **3.1.2. Determinación simultánea de residuos hormonales en aguas depuradas usando cromatografía líquida de ultra resolución con espectrometría de masas en tándem.**

Teniendo en cuenta que el consumo de hormonas esteroideas como fármacos se ha incrementado de manera exponencial en los últimos años y, los potenciales efectos de las hormonas sobre el medio acuático, es necesario desarrollar metodologías de análisis que permitan el control de este tipo de compuestos. Dicho control debe realizarse en muestras ambientales, pero sobre todo en aquellos puntos críticos donde se produzca la entrada de hormonas al medio, pues en ellos el impacto es mayor. Las aguas residuales constituyen uno de estos puntos críticos ya que su contenido en hormonas es considerablemente más alto que el de las aguas ambientales. Además, el estudio de la eficiencia de eliminación de las diferentes tecnologías de depuración es un objetivo primordial en el análisis medioambiental, pues permite conocer aquellos tratamientos que proporcionan mejores rendimientos y efluentes más limpios y, consecuentemente, menos tóxicos.

Sin embargo, la complejidad de las muestras de aguas residuales hace necesario el desarrollo de metodologías sensibles y selectivas que permitan extraer, preconcentrar y determinar las hormonas presentes en las aguas, eliminando a su vez la mayor cantidad posible de interferencias. En este sentido, en esta publicación se presenta la optimización de una metodología basada en extracción en fase sólida y determinación por cromatografía líquida de ultra resolución con detección por espectrometría de masas en tándem (SPE-UHPLC-MS/MS). Este trabajo es

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una ampliación del realizado anteriormente (apartado 3.1.1) pues se aumentó el número de hormonas esteroideas estudiadas de cuatro a ocho, añadiendo compuestos de las familias de los estrógenos (dietilestilbestrol y estrona), los andrógenos (testosterona) y progestágenos (acetato de megestrol y levonorgestrel).

Para ello, primero se optimizó la detección por espectrometría de masas, determinando los iones precursores y de fragmentación de los compuestos en estudio, así como las condiciones óptimas de los parámetros de la detección. La cromatografía líquida de ultra resolución consiste en una mejora de la cromatografía líquida de alta resolución en la que se trabaja a presiones superiores, lo que se traduce en análisis más cortos, menor uso de disolventes y picos cromatográficos mejor definidos. En el caso del presente trabajo, se consigue realizar la separación cromatográfica y determinación de los compuestos en estudio en 5,5 minutos, lo cual es muy beneficioso, pues permite el análisis de gran cantidad de muestras en un espacio corto de tiempo.

A continuación, se procedió a realizar la optimización de las variables del método de extracción, siguiendo un método experimental basado en diseños factoriales a través de los cuales se puede evaluar fácilmente cómo afectan las diferentes variables al método de extracción.

El método desarrollado presenta una excelente linealidad, así como límites de detección muy bajos, en el rango de 0,15 a 9,35 ng·L<sup>-1</sup>, indicados para el análisis de muestras medioambientales. Además, las recuperaciones del método de extracción fueron muy buenas, superando el 70% de extracción para todos los compuestos a excepción del dietilestilbestrol. En la determinación mediante espectrometría de masas

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con ionización de electrospray se presenta un problema derivado de la naturaleza de la muestra conocido como efecto matriz, por lo que se hace necesario evaluarlo. En este caso se comprobó que el método de extracción era bastante selectivo y no se extraían interferencias que produjeran problemas de ionización de la señal, pues los efectos matrices calculados presentaron unos valores de  $\pm 20\%$ .

Seguidamente, el método desarrollado se aplicó a muestras reales procedentes de estaciones depuradoras de aguas residuales de la isla de Gran Canaria, tanto de la entrada como de la salida de éstas. Se realizaron dos campañas de muestreo en dos estaciones depuradoras que aplicaban diferentes métodos de tratamiento. Una de ellas utilizaba un método tradicional de lodos activados, mientras que la otra usaba un tratamiento de biorreactor de membrana. Las hormonas estudiadas se detectaron en concentraciones que variaron de 9 a 30  $\text{ng}\cdot\text{L}^{-1}$ , apareciendo algunas de ellas (testosterona y dietilestilbestrol) en todos los muestreos.

El artículo fue publicado en la revista *Journal of Analytical Methods in Chemistry* (2013), ID 210653:1-8 en modalidad de acceso abierto (*Open-access*).





### Research Article

# Simultaneous Determination of Hormonal Residues in Treated Waters Using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry

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In the last years, hormone consumption has increased exponentially. Because of that, hormone compounds are considered emerging pollutants since several studies have determined their presence in water influents and effluents of wastewater treatment plants (WWTPs). In this study, a quantitative method for the simultaneous determination of oestrogens (estrone, 17 $\beta$ -estradiol, estriol, 17 $\alpha$ -ethinylestradiol, and diethylstilbestrol), androgens (testosterone), and progestogens (norgestrel and megestrol acetate) has been developed to determine these compounds in wastewater samples. Due to the very low concentrations of target compounds in the environment, a solid phase extraction procedure has been optimized and developed to extract and preconcentrate the analytes. Determination and quantification were performed by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The method developed presents satisfactory limits of detection (between 0.15 and 9.35 ng·L<sup>-1</sup>), good recoveries (between 73 and 90% for the most of compounds), and low relative standard deviations (under 8.4%). Samples from influents and effluents of two wastewater treatment plants of Gran Canaria (Spain) were analyzed using the proposed method, finding several hormones with concentrations ranged from 5 to 300 ng·L<sup>-1</sup>.

## 1. Introduction

In general, it is supposed that more than 100,000 different chemical compounds can be introduced in the Environment, many of them in very small quantity. However, a lot of these compounds are not included as pollutants in the legislation. Although 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 is 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].

Hormones are one of the most potent endocrine disrupting compounds as well as are considered also as emerging pollutants. Hormones can be differentiated in oestrogens, androgens, and progestogens. Some of them have limits in their use, but not a specific legislation [4].

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 [5].

The steroid hormones help controlling the metabolism, inflammations, immunological functions, water and salt balance, sexual development, and the capacity of withstanding illnesses [6]. 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 [7].

A significant quantity of consumed oestrogens leaves the organism through excretions. For example,  $17\beta$ -estradiol (E2) is oxidized rapidly, becoming an estrone (E1) that can turn into estriol afterwards (E3). Besides, the  $17\alpha$ -ethinyl estradiol (EE) is excreted as conjugated [8].

With regard to emission sources, in the first place are the wastewater treatment plants (WWTPs) [9], and secondarily, cattle waste such as those leachates from dung and uncontrolled dumping [10]. Several studies made in the WWTPs have reported that the treatment plants are capable of eliminating around 60% of hormones [11–13].

The identification of hormone residues in environment is of special interest because knowledge of these compounds is a requirement to take measures in order to regulate and minimize their environmental impact.

However, 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 [14–17]. 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) [18–20].

The objective of this study is to develop a rapid and simple procedure of extraction, preconcentration, and determination of four steroid oestrogens (estrone (E1),  $17\beta$ -estradiol (E2), estriol (E3) and  $17\alpha$ -ethinylestradiol (EE)), one non-steroidal oestrogen, the diethylstilbestrol (DES), one androgen, the testosterone (TES) and two synthetic progestogens, norgestrel (NOR) and megestrol acetate (MGA) (Table 1), based on solid phase extraction and ultrahigh performance liquid chromatography-tandem mass spectrometry (SPE-UHPLC-MS/MS). The developed method was 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 presented different methods of wastewater treatments: WWTP 1 presented a traditional method based on activated sludge, while WWTP 2 used 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  $1000 \text{ mg}\cdot\text{L}^{-1}$  of each analyte were prepared by

dissolving the compound in methanol, and the solutions were stored in glass-stoppered bottles at  $4^\circ\text{C}$  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 and August of 2012. WWTP 1 used a conventional activated sludge treatment system, while WWTP 2 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  $0.65 \mu\text{m}$  membrane filters (Millipore, Ireland). The samples were stored in the dark at  $4^\circ\text{C}$  and extracted within 48 hours.

**2.3. Instrumentation.** For the SPE optimization, the instrument used was an ultrahigh 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  $25 \mu\text{L}$  syringe and a tray to hold 2 mL vials, and an 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 kV in positive mode, and  $-2 \text{ kV}$  in negative mode, the source temperature was  $150^\circ\text{C}$ , the desolvation temperature was  $500^\circ\text{C}$ , and the desolvation gas flow rate was  $1000 \text{ L/hr}$ . 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  $1 \text{ mg}\cdot\text{L}^{-1}$  standard solution of each analyte into the detector at a flow rate of  $10 \mu\text{L}\cdot\text{min}^{-1}$ .

**2.4. Chromatographic Conditions.** For the SPE optimization, the analytical column was a  $50 \text{ mm} \times 2.1 \text{ mm}$ , ACQUITY UHPLC BEH Waters  $\text{C}_{18}$  column with a particle size of  $1.7 \mu\text{m}$  (Waters Chromatography, Barcelona, Spain) operating at a temperature of  $30^\circ\text{C}$ . Analytes separation was carried out employing the following gradient: starting 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

TABLE 1: List of hormonal compounds, pK<sub>a</sub> values, chemical structure, and retention times.

Compound	pK <sub>a</sub> [21]	Structure	t <sub>R</sub> (min)
E3 Estriol	10.3		0.96
E2 17β-estradiol	10.3		2.18
EE 17α-ethinylestradiol	10.3		2.23
E1 Estrone	10.3		2.20
DES Diethylstilbestrol	10.2		2.35
TES Testosterone	15.1		2.40
MGA Megestrol acetate			3.06
NOR Levonorgestrel	13.1		2.63

minutes. Therefore, the analysis took 9 minutes at a flow of 0.5 mL·min<sup>-1</sup>.

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 and methanol, adjusted with a buffer consisting in 0.1% v/v ammonia, and 15 mM of ammonium acetate. The analysis was performed in gradient mode at a flow rate of 0.3 mL·min<sup>-1</sup>. The gradient started at 50 : 50 (v/v) mixture of water : methanol, which changed to 25 : 75 (v/v) in 3 minutes, and returned to 50 : 50 in 1 minute more. Finally, the gradient stayed calibrating for another 1.5 minutes more. The sample volume injected was 5 μL.

### 3. Results and Discussion

3.1. Optimization of Solid-Phase Extraction (SPE). There are a number of parameters that affect SPE procedure such as

type of sorbent, pH, ionic strength, sample and desorption volumes, and wash step. To optimize these parameters, it used Milli-Q water spiked with a solution of fluorescence oestrogens (estriol, 17β-estradiol, and 17α-ethinylestradiol) to obtain a final concentration of 250 μg·L<sup>-1</sup>.

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<sub>18</sub> (both from Waters, Madrid, Spain), and BondElut ENV (from Agilent, Madrid, Spain). Keeping other parameters fixed (ionic strength of 0%, sample volume of 100 mL), the cartridges were studied at three different pHs (5, 8, and 11). From the results obtained, it can be observed that the better signals are found for SepPak C<sub>18</sub> cartridge (Figure 1).

After choosing the optimum cartridge, we used an initial experimental design of 2<sup>3</sup>, to study the influence of pH, ionic

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)	Quantification ion, $m/z$ (collision potential, V)
E3	287.2	-65 V (ESI -)	171.0 (37)	145.2 (39)
E2	271.2	-65 V (ESI -)	145.1 (40)	183.1 (31)
EE	295.2	-60 V (ESI -)	145.0 (37)	158.9 (33)
E1	269.2	-65 V (ESI -)	145.0 (36)	143.0 (48)
DES	267.1	-50 V (ESI -)	237.1 (29)	251.1 (25)
TES	289.2	38 V (ESI +)	187.0 (18)	104.0 (21)
MGA	385.5	30 V (ESI +)	267.3 (15)	224.2 (30)
NOR	313.2	38 V (ESI +)	109.0 (26)	245.1 (18)

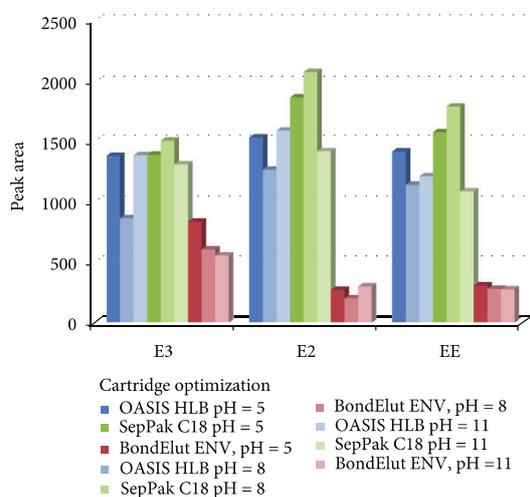


FIGURE 1: Optimization of SPE cartridges.

strength and sample volume over extraction process. The experimental design was obtained using Statgraphics Plus software 5.1 and the statistics study was done with IBM SPSS Statistics 19. We assessed two levels and three parameters: pH (3 and 8), ionic strength (0 and 30% of NaCl), and sample volume (50 and 250 mL), to obtain the influence of each parameter and the variable correlation to each other. In this study, it is 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% of NaCl for ionic strength and 50, 100 y 250 mL for sample volume) was used. Figure 2 shows the response surface obtained for the estriol and  $17\alpha$ -ethinyloestradiol. The results obtained showed 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. Because of that, a solution without salt addition and 250 mL of sample volume was chosen. 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<sub>18</sub> 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. In Figure 3, a chromatogram with the optimum conditions is shown, where the peaks of all compounds in their corresponding transitions can be observed.

**3.2. Analytical Parameters.** Because of the SPE optimization, that was done only with fluorescent compounds (estriol,  $17\beta$ -estradiol, and  $17\alpha$ -ethinyloestradiol), it was necessary to study the recoveries of all the hormonal compounds, using the optimized SPE-UHPLC-MS/MS method. All the compounds under study showed good recoveries, over 73%, except the diethylstilbestrol, with a recovery of 50.7%.

A 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  $100 \mu\text{g}\cdot\text{L}^{-1}$ . 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.

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 0.15 to  $9.35 \text{ ng}\cdot\text{L}^{-1}$  and the LOQs ranged from 0.49 to  $31.18 \text{ ng}\cdot\text{L}^{-1}$ .

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 8.4% in all cases, indicating a

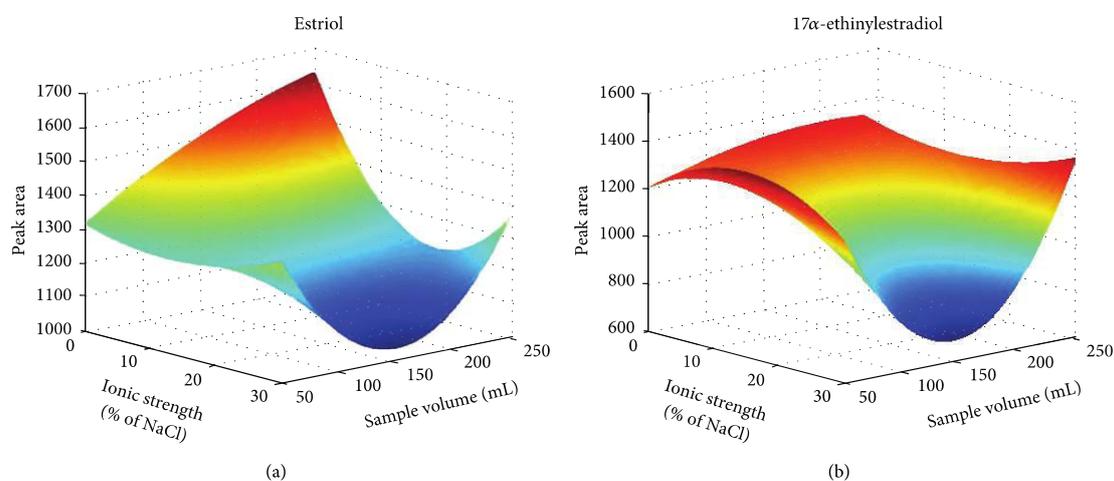


FIGURE 2: Effect of ionic strength and sample volume on the SPE extraction for estriol and 17 $\alpha$ -ethinylestradiol.

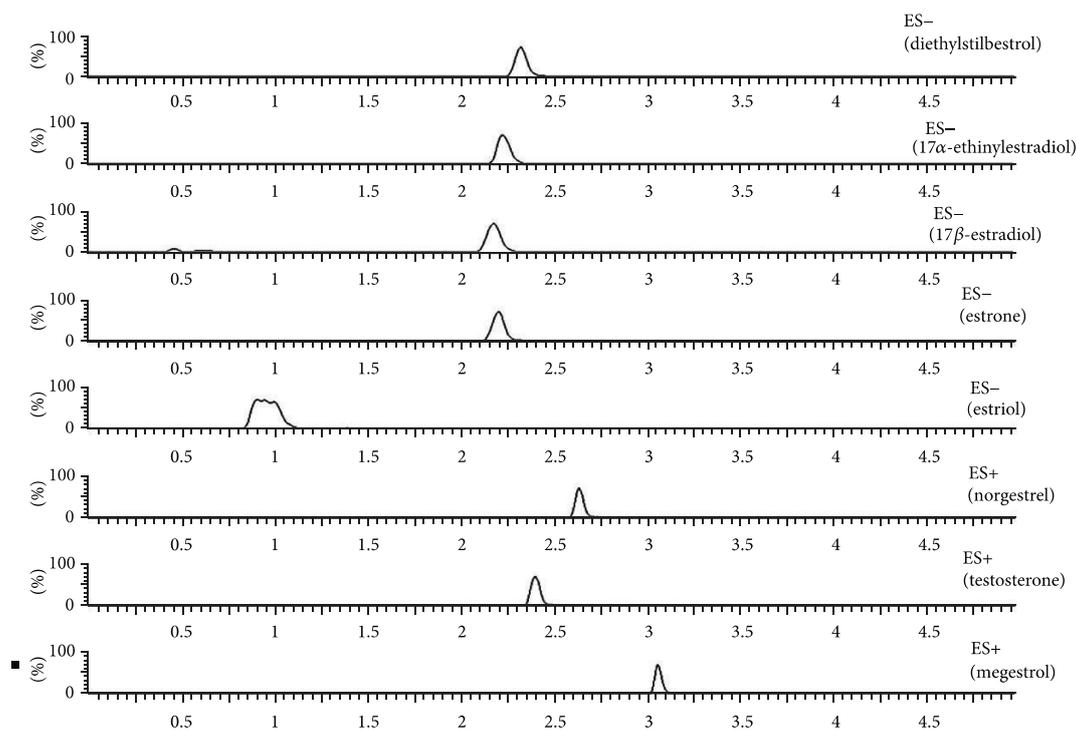


FIGURE 3: MRM chromatograms of a spiked sample ( $250 \mu\text{g}\cdot\text{L}^{-1}$ ) with all analytes after SPE process.

good repeatability. Table 3 shows the analytical parameters obtained for all compounds analysed.

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 [22]. To

evaluate the relative signal enhancement or suppression in the samples, the algorithm by Vieno et al. [23] was used, as following:

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

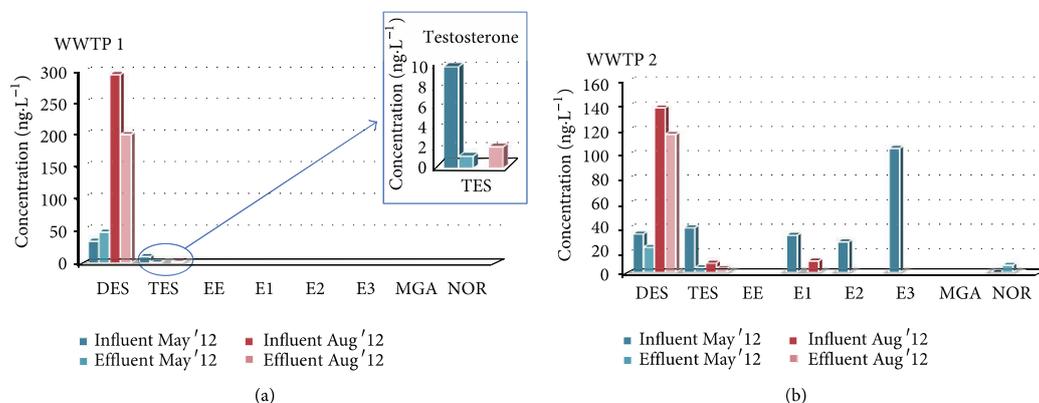


FIGURE 4: Concentrations of target compounds in sewage samples from both wastewater treatment plants (WWTPs).

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

Compound	RSD <sup>a</sup> (%) <i>n</i> = 6	LOD <sup>b</sup> (ng·L <sup>-1</sup> )	LOQ <sup>c</sup> (ng·L <sup>-1</sup> )	Recovery (%) <i>n</i> = 6	Matrix effect (%)
E3	6.53	9.35	31.2	80.5 ± 5.3	2.96
E2	8.37	2.53	8.44	89.7 ± 7.5	1.33
EE	7.25	0.51	1.71	90.6 ± 6.5	-12.6
E1	6.81	2.60	8.66	78.7 ± 5.4	15.4
DES	6.93	0.64	2.14	50.7 ± 3.5	4.48
TES	6.77	1.49	4.95	83.8 ± 5.7	17.1
MGA	7.18	0.15	0.49	73.7 ± 5.3	13.5
NOR	7.38	2.11	7.04	88.9 ± 6.5	17.2

<sup>a</sup>Relative standard derivation.

<sup>b</sup>Detection limits, calculated as signal-to-noise ratio of three times.

<sup>c</sup>Quantification limits, calculated as signal-to-noise ratio of ten times.

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.

Suppression effect, between 13 and 17%, was observed for estrone, testosterone, norgestrel, and megestrol acetate. For estriol, 17 $\beta$ -estradiol, and diethylstilbestrol, the signal suppressions were very low, under 5%. Only 17 $\alpha$ -ethinylestradiol showed a signal enhancement of 12.6%. The results obtained are showed in Table 3 and they are in accordance with those reported in similar studies [19, 24].

**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). Figure 4 shows the results obtained. It can be observed that in the WWTP 1, not all compounds were detected, only diethylstilbestrol and testosterone, in concentration that ranging between 35 and 300 ng·L<sup>-1</sup> and 1.2 and 9.95 ng·L<sup>-1</sup>, respectively, for influent samples. The

concentrations of diethylstilbestrol at the effluent increased in the first sampling and diminished in the second. The behaviour of testosterone in the effluent samples was the opposite.

However, for WWTP 2 a higher number of compounds were detected. For the influents samples, the highest concentrations were between 100 and 140 ng·L<sup>-1</sup> for estriol and diethylstilbestrol, while the rest of compounds (testosterone, estrone and 17 $\beta$ -estradiol) were detected at concentrations between 20 and 40 ng·L<sup>-1</sup>. The concentrations at the effluent for diethylstilbestrol diminished up to 110 ng·L<sup>-1</sup> and 5 ng·L<sup>-1</sup> for testosterone. The rest of compounds were not detected at the effluent samples. Only the concentration of norgestrel (about 6 ng·L<sup>-1</sup>) increased during the wastewater treatment process.

## 4. Conclusions

An analytical method for the simultaneous extraction, pre-concentration, and determination of oestrogens (estrone, 17 $\beta$ -estradiol, estriol, 17 $\alpha$ -ethinylestradiol, and diethylstilbestrol) androgens (testosterone) and progestogens (norgestrel

and megestrol acetate) 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 0.15 to 9.35 ng·L<sup>-1</sup>. In addition, the method presented high recoveries, up to 90%, for the majority of compounds and RSD lower than 9%.

The application of the method to samples from two different WWTPs showed that the concentrations of hormones found, ranged from 5 to 300 ng·L<sup>-1</sup>, and some of them (diethylstilbestrol and testosterone) were detected in all the wastewater samples, and other, like estrone or 17β-estradiol, only in some samples. In view of the obtained results about influent and effluent samples, it can be determined that the membrane bioreactor system is quite effective to degrade these compounds. However, it is difficult to obtain a conclusion about the activate sludge treatment effectivity owing to the small quantity of compounds detected.

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#### **3.1.3. Método de extracción en fase sólida en modalidad *on-line* acoplada a UHPLC-MS/MS para la determinación de hormonas esteroideas en aguas depuradas procedentes de estaciones depuradoras de aguas residuales.**

Las tendencias actuales en el campo de los métodos de extracción y preconcentración se centran en el desarrollo de metodologías que se aproximen a la sostenibilidad y la química verde. Desde los primeros métodos de extracción en los que se producía la paradoja de que éstos podían ser más contaminantes para el medio que los propios contaminantes analizados, se ha perseguido continuamente la minimización del uso de disolventes orgánicos, así como de volúmenes de muestras, lo que ha redundado en procedimientos más simples, más precisos y más cortos. Además, la automatización de las metodologías se ha revelado como un avance fundamental ya que permite minimizar de manera sustancial todos aquellos errores derivados de la manipulación de muestras. Todo esto ha permitido que actualmente se estén desarrollando métodos automatizados, que además puedan ser acoplados a los sistemas de separación y determinación, lo cual producirá menores tiempos de análisis y mejores reproducibilidades.

En el presente trabajo se presenta el desarrollo de una metodología de extracción en fase sólida en modalidad en línea (*on-line*) acoplada a un sistema de cromatografía líquida de ultra resolución con detección de espectrometría de masas en tándem. En el sistema utilizado en este trabajo se utilizan dos columnas de extracción en fase sólida que trabajan en paralelo, ya que mientras en una se produce la extracción, la otra se

### Capítulo 3. Parte experimental y resultados

encuentra en fase de lavado y acondicionamiento para la siguiente muestra, lo que minimiza también los tiempos de espera entre análisis.

Además, se aumentó el número de hormonas estudiadas a quince, cinco estrógenos, cuatro progestágenos, tres andrógenos y tres glucocorticoides. La novedad principal de este método, junto al uso de un procedimiento de extracción *on-line*, reside en el estudio de un método analítico para la determinación simultánea de hormonas esteroideas de cuatro familias diferentes, lo que hace que éste sea muy adecuado para evaluar la presencia de este tipo de contaminantes, en muestras líquidas medioambientales.

El aumento del número de compuestos en estudio supone una dificultad añadida debido a la similitud de las estructuras de los compuestos estudiados. Este hecho hace que el estudio de las condiciones de separación y detección sea muy importante ya que se debe conseguir la determinación de todos los analitos de manera sensible a la vez que deben optimizarse los parámetros de la ionización y determinación en el detector de espectrometría de masas para que éstos sean adecuados para todos los compuestos.

Una vez realizada la optimización del proceso de separación y determinación se optimizaron las variables que afectan al proceso de extracción en fase sólida *on-line*, como tipo de columna de extracción, composición de la fase de carga y de la fase de lavado y pH de la muestra. Tras la optimización de todo el proceso de análisis se obtuvo un método altamente sensible y reproducible, en el que el tiempo de extracción, separación y determinación de los quince compuestos hormonales fue inferior a 15 minutos. Los límites de detección del método se situaron

### Capítulo 3. Parte experimental y resultados

entre 0,5 y 13,2 ng·L<sup>-1</sup> para todos los compuestos y las recuperaciones obtenidos fueron muy satisfactorias, en el rango de 50 a 90%.

Tras el desarrollo del método, éste fue aplicado satisfactoriamente a tres muestras de efluentes de estaciones depuradoras de la isla de Gran Canaria. Una de las depuradoras trata el agua de una zona rural situada en el norte de la isla y presenta una tecnología de biorreactor de membrana, mientras que las otras dos EDARs se basan en un tratamiento convencional de lodos activos y depuran las aguas residuales de dos importantes aglomeraciones urbanas de la zona este de la isla.

En los efluentes de las EDARs basadas en lodos activos se detectaron dos estrógenos, un andrógeno y un progestágeno en concentraciones que variaron desde 3,1 a 52,8 ng·L<sup>-1</sup>, mientras que en el efluente de la EDAR basada en el biorreactor de membrana no se detectó ninguna de las hormonas en estudio.

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## An on-line solid phase extraction method coupled with UHPLC-MS/MS for the determination of steroid hormone compounds in treated water samples from waste water treatment plants

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An on-line solid phase extraction coupled with ultra-high performance liquid chromatography in tandem with mass spectrometry (SPE-UHPLC-MS/MS) method for the determination of fourteen hormones (four oestrogens, three androgens, four progestogens and three corticosteroids) in waste water samples has been developed. All of the parameters involved in the on-line extraction process have been optimized: type of cartridge, sample volume, loading solvent, solvent of the wash step and the pH of the sample. Moreover, the chromatographic separation and all of the parameters involved in the detection by mass spectrometry have been studied too. The developed method allows for complete analysis (extraction and identification of the analytes) in 14.5 minutes. The method is selective, with satisfactory relative standard deviations (lower than 15% in most cases) and limits of detection and quantification that ranged from 0.5 to 13.2 ng L<sup>-1</sup> and from 1.66 to 44 ng L<sup>-1</sup>, respectively. The recoveries were acceptable for most compounds for effluent samples from different waste water treatment plants (between 50 and 90%). The proposed method has been applied to study effluent samples from three waste water treatment plants from Gran Canaria (Spain). Four steroid hormones of different families have been detected at concentrations ranging from 3.1 to 52.8 ng L<sup>-1</sup>.

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### 1. Introduction

In recent years, endocrine disrupting compounds (EDCs) have garnered increasing attention from the international community. Changes in aquatic biota, such as hermaphroditism, feminization, inhibition of locomotion and aggressive behaviour or changes in fertility or vitellogenin, are produced by these types of emerging pollutants, which have been discussed in several studies.<sup>1-4</sup> Among the EDCs, we can consider steroid hormones as a wide group that can be divided into four subgroups: oestrogens, androgens, progestogens and corticosteroids. Oestrogens, such as 17β-estradiol (E2), estrone (E1) and oestriol (E3), are female hormones that are essential to the menstrual cycle of women. Natural and synthetic oestrogens are used in both human and veterinary medicine with the main medical application being birth control. The most used synthetic oestrogen for birth control is 17α-ethinylloestradiol (EE). Progestogens, also called gestagens, are characterized by their basic 21-carbon skeleton and their main function is to maintain the pregnancy, although they are expressed in several phases of the menstrual cycle. Consequently, progestogens are also used as hormonal contraceptives that can be combined

with oestrogens. In the last decade, the consumption of oestrogens, with and without combination with progestogens, has greatly increased. In fact, currently, 100 million women are active users of combined hormonal contraceptives worldwide.<sup>5</sup> Alternatively, androgens are frequently used by some sportsmen to increase their strength, mass and muscular size. Nevertheless, the doses are higher than the doses used in hormone replacement therapies; in consequence, serious side effects can appear, such as testicular atrophy, sterility, gynecomastia in males and ovulation inhibition, hirsutism, alopecia and acne in females.<sup>6</sup> Finally, corticosteroids are synthesized in the adrenal cortex of vertebrates and are involved in many physiological processes. Corticosteroids are divided into mineralocorticoids and glucocorticoids and these substances can be artificially synthesized for therapeutic applications due to their anti-inflammatory properties and immunosuppressive effects on metabolism. Corticosteroids are illegal in the EU for fattening purposes as legislated in the 96/22/EC directive.<sup>7</sup>

A significant quantity of consumed hormones exit organisms through excretions.<sup>8,9</sup> For this reason, most publications agree that waste water treatment plants (WWTPs) are the principal sources of EDC release into the environment.<sup>10</sup> The presence of hormone compounds in the effluents of WWTPs is due to their incomplete degradation by treatment processes, which produces an alarming contamination in aquatic

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**Table 1** List of hormone compounds, surrogate standards,  $pK_a$  values, and retention times

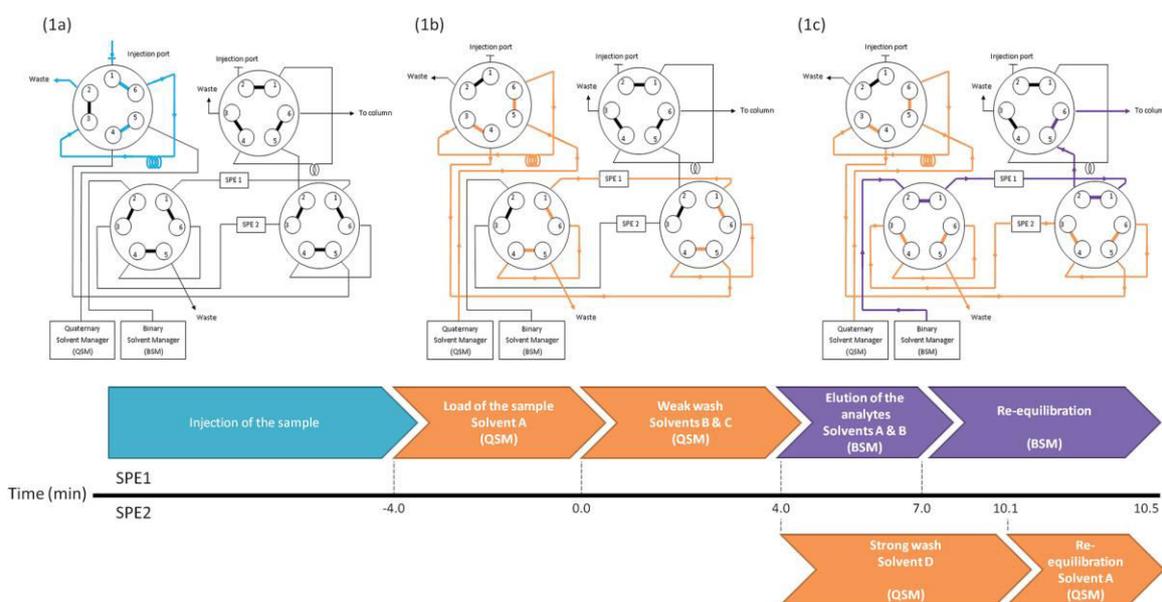
Type of hormone	Abbreviation	Compound	Surrogate standard	$pK_a^{35}$	$t_R$ (min)
Oestrogens	E3	Estriol	Estrone D2	10.3	6.50
	E2	17 $\beta$ -estradiol		10.3	7.07
	E1	Estrone		10.3	7.07
	DES	Diethylstilbestrol		10.2	7.08
Progestogens	NORET	Norethisterone	Progesterone D9	13.1	7.05
	NOR	Norgestrel		13.1	7.20
	MGA	Megestrol acetate		—	7.32
	PRO	Progesterone		—	7.40
Androgens	BOL	Boldenone	Testosterone D3	15.1	7.00
	NAN	Nandrolone		15.1	7.05
	TES	Testosterone		15.1	7.15
Corticosteroids	PRED	Prednisone	Progesterone D9	12.4	6.60
	COR	Cortisone		12.4	6.63
	PREDNL	Prednisolone		12.5	6.73

environments.<sup>11–13</sup> The compound concentrations found in the environment are in the range of  $\text{ng L}^{-1}$ <sup>12,14,15</sup> because of the low doses of these drugs, their catabolism by humans and most of them degrade in WWTPs.

Because of the low concentration of steroid hormones in the environment, it is necessary to develop sensitive methods for extraction, preconcentration and identification of hormones in water samples. Solid phase extraction (SPE) is a widespread method of extraction used to isolate and preconcentrate emerging pollutants from aqueous matrices.<sup>16–18</sup> Some authors have reported extraction of oestrogens, androgens, progestogens and corticosteroids from WWTP samples using this method in the last decade.<sup>18–21</sup> The separation and identification techniques used more often in recent years have been liquid chromatography with mass spectrometry detection (LC-

MS)<sup>22</sup> and liquid chromatography in tandem with mass spectrometry detection (LC-MS/MS).<sup>19,20,23,24</sup> These techniques allow the identification of hormones without a derivatization step, which is needed when using GC-MS.<sup>25–27</sup> On-line SPE methods have been developed in recent years and present advantages over off-line SPE methods, such as lower sample handling and analysis time. On-line SPE coupled to HPLC-MS/MS and UHPLC-MS/MS provides a highly sensitive and specific method for steroid hormone detection in water samples.<sup>20,24,28</sup>

In this study, an on-line SPE process coupled with liquid chromatography in tandem with mass spectrometry detection system has been developed for the determination of fourteen steroid hormones belonging to four subgroups (Table 1). All of the conditions involved in the extraction, separation and identification processes have been optimized using the effluent



**Fig. 1** Positions of the valves and solvents in different on-line SPE events.

Table 2 Gradient used in Binary and Quaternary solvent Managers<sup>a</sup>

Time (min)	Binary solvent manager			Quaternary solvent manager					
	Flow (mL min <sup>-1</sup> )	A (%)	B (%)	Flow (mL min <sup>-1</sup> )					
				OASIS HLB	XBridge C <sub>18</sub>	A2 (%)	B2 (%)	C (%)	D (%)
0.0	0.30	80	20	2.00	2.00	100	0	0	0
3.8	0.30	80	20	2.00	0.01	0	100	0	0
4.1	0.30	80	20	2.00	0.01	0	100	0	0
7.0	0.30	0	100	2.00	2.00	0	0	0	100
8.0	0.30	0	100	2.00	2.00	100	0	0	0
10.5	0.30	80	20	2.00	2.00	100	0	0	0

<sup>a</sup> A: water + 0.1% NH<sub>3</sub>, A2: water + 0.05% formic acid, B: methanol, B2: water, C: methanol, D: acetone : hexane : methanol (1 : 1 : 1).

from a tertiary treatment used at a waste water treatment plant (WWTP1). The developed method has been applied to study effluent samples from three WWTPs (WWTP2, WWTP3 and WWTP4) located in Gran Canaria Island (Spain) which use different water treatments. WWTP2 uses a membrane bioreactor for biological treatment and WWTP3 and WWTP4 use the traditional activated sludge treatment.

## 2. Materials and methods

### 2.1. Reagents

All of the hormone compounds were purchased from Sigma-Aldrich (Madrid, Spain). The purities of all compounds under study are over 99.0%. Three surrogate standards were used: estrone D2 and progesterone D9 from CDN Isotopes Inc. (Quebec, Canada) and testosterone D3 from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Stock solutions

containing 1000 mg L<sup>-1</sup> of each analyte were prepared by dissolving the compound in methanol and stored in glass-stoppered bottles at -20 °C prior to use. Working aqueous standard solutions were prepared daily. Ultrapure water used in the on-line SPE process was obtained by using a Milli-Q system (Millipore, Bedford, MA, USA). HPLC-grade methanol, LC-MS methanol, LC-MS water, HPLC-grade *n*-hexane and HPLC-grade acetone, as well as ammonia, ammonium acetate and acetic acid 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 four waste water treatment plants located in Gran Canaria in May of 2014 and January of 2015. WWTP1 samples were from the effluent of the tertiary process and were used to optimize the on-line SPE method. WWTP2 has a population equivalent of 7000 and uses

Table 3 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)	Confirmation – Quantification ion ratio
E3	287.2	-65 V (ESI-)	171.0 (37)	145.2 (39)	0.19
PRED	359.3	30 V (ESI+)	147.0 (15)	237.0 (20)	0.25
COR	361.3	30 V (ESI+)	163.0 (25)	121.0 (45)	0.10
PREDNL	361.3	20 V (ESI+)	147.1 (20)	173.1 (25)	0.39
BOL	287.2	30 V (ESI+)	121.0 (28)	135.1 (15)	0.59
NAN	275.2	35 V (ESI+)	109.1 (20)	83.0 (30)	0.53
NORET	299.2	30 V (ESI+)	109.1 (25)	91.0 (40)	0.59
E2	271.2	-65 V (ESI-)	145.1 (40)	183.1 (31)	0.23
E1	269.2	-65 V (ESI-)	145.0 (36)	143.0 (48)	0.22
DES	267.1	-50 V (ESI-)	237.1 (29)	251.1 (25)	0.91
TES	289.2	38 V (ESI+)	97.0 (22)	109.0 (21)	0.80
NOR	313.2	38 V (ESI+)	109.0 (26)	245.1 (18)	0.56
MGA	385.5	30 V (ESI+)	267.3 (15)	224.2 (30)	0.66
PRO	315.3	30 V (ESI+)	97.0 (18)	109.1 (25)	0.86
Deuterated Compound	Precursor ion (m/z)	Cone voltage (ion mode)	Quantification ion, m/z (collision potential, V)	Confirmation ion, m/z (collision potential, V)	Confirmation – quantification ion ratio
E1-d2	271.2	70 V (ESI-)	147.1 (30)	145.1 (35)	0.12
TES-d3	292.2	35 V (ESI+)	97.1 (25)	109.1 (20)	0.80
PRO-d9	324.3	35 V (ESI+)	100.1 (20)	113.1 (20)	0.56

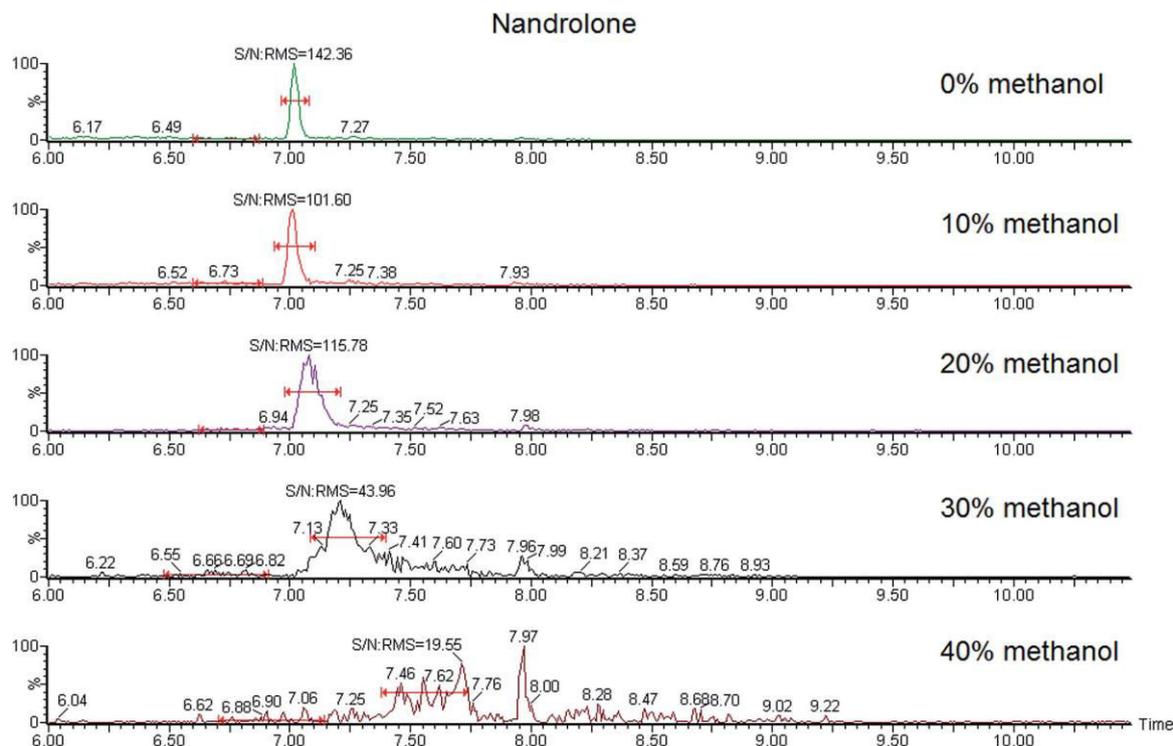


Fig. 2 Effect of methanol of the wash step in the peak detection of nandrolone, including the *S/N* ratio.

a membrane bioreactor treatment system. WWTP3 and WWTP4 treat the water from big urban areas of Gran Canaria (population equivalents of 60 000 and 130 000, respectively) and use an activated sludge treatment. The samples were collected in 2 L amber glass bottles that were rinsed beforehand with methanol and ultrapure water. Samples were acidified to inhibit microbial activity and purified through filtration with fibreglass filters and then with 0.22  $\mu\text{m}$  membrane filters (Millipore, Ireland). The samples were stored in the dark at 4  $^{\circ}\text{C}$  and they were analyzed within 48 hours.

### 2.3. On-line SPE-UHPLC system

The on-line SPE-UHPLC-MS/MS system used was obtained from Waters (Waters Chromatography, Barcelona, Spain). This system consisted of a binary solvent manager (BSM) pump for the chromatographic separation, a quaternary solvent manager (QSM) pump to perform the extraction process, an autosampler capable of injecting volumes up to 5000  $\mu\text{L}$  per injection, a column manager and a triple quadrupole detector (TQD). Solid phase extraction was performed using OASIS HLB (2.1  $\times$  30 mm, 20  $\mu\text{m}$ ) and XBridge  $\text{C}_{18}$  (2.1  $\times$  30 mm, 10  $\mu\text{m}$ ) SPE columns (Waters Chromatography, Barcelona, Spain) followed by elution of the analytes with the chromatographic mobile phase and finally, separation was achieved in the analytical column placed in the column manager.

A scheme of the on-line SPE process is shown in Fig. 1. First, the autosampler injects a volume of up to 5000  $\mu\text{L}$  into valve 2

and the sample is placed in the loop (Fig. 1a). Next is the loading phase (solvent A of the QSM) where the sample is loaded into the SPE column 1. After loading, solvents B and C of the QSM perform the wash step to eliminate interferences in the sample (Fig. 1b). After the wash step, a change in the valves allows for column 2 to be strongly washed with a mixture of organic solvents (solvent D of the QSM) while SPE column 1 is eluted with the chromatographic mobile phase of the binary solvent manager (BSM) (Fig. 1c). After the strong wash and during the chromatographic separation, SPE column 2 is conditioned and equilibrated with the load phase (solvent A of the QSM) to prepare it for the next extraction.

In this system the solvent pumps have different purposes. The quaternary solvent manager (QSM) is used to load the sample into the SPE column, a weak wash of the SPE column to eliminate interferences and to strongly wash the SPE columns to eliminate any analyte retention. The binary solvent manager serves for elution of the analytes to the separation column and chromatographic analyses.

### 2.4. Chromatographic and mass spectrometry conditions

**2.4.1. Chromatographic separation.** The analytical column used was a 50 mm  $\times$  2.1 mm, ACQUITY UHPLC BEH Waters  $\text{C}_{18}$  column with a particle size of 1.7  $\mu\text{m}$  (Waters Chromatography, Barcelona, Spain) operating at a temperature of 30  $^{\circ}\text{C}$ . The injection volume was 2 mL of the sample and the mobile phases were water, with 0.1% v/v ammonia (A) and methanol (B). The

Table 4 Analytical parameters of target analytes for every sample studied

Compound	LOD <sup>a</sup> (ng L <sup>-1</sup> )	Effluent WWTP1				Effluent WWTP2			
		100 ng L <sup>-1</sup>		500 ng L <sup>-1</sup>		100 ng L <sup>-1</sup>		500 ng L <sup>-1</sup>	
		Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6	Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6	Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6	Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6
Diethylstilbestrol	13.2	44.3	7.3	42.3	14.7	44.3	14.6	51.9	4.7
17β-estradiol	8.5	88.8	26.4	104.0	7.0	126.7	14.6	112.8	6.2
Estrone	4.1	75.1	15.1	81.6	8.8	75.5	15.8	82.6	5.3
Estriol	4.5	76.8	5.2	69.7	17.1	58.6	16.9	78.5	11.0
Norgestrel	1.6	34.5	8.6	36.7	11.6	42.5	8.1	48.4	6.1
Testosterone	1.0	53.1	6.9	52.3	3.7	69.7	6.3	74.4	2.8
Megestrol acet.	1.2	138.7	6.8	154.4	10.8	153.6	11.4	195.9	3.7
Prednisone	9.2	61.7	11.5	60.7	5.0	97.5	9.8	82.3	12.0
Prednisolone	6.1	95.2	9.4	100.0	8.7	133.0	7.3	120.4	4.8
Cortisone	2.1	69.5	7.3	66.3	3.2	88.7	13.1	86.9	6.0
Boldenone	0.7	61.1	4.5	67.5	2.7	95.7	6.3	106.9	2.1
Norethisterone	2.3	42.7	2.9	44.3	3.3	73.3	9.5	76.9	2.5
Nandrolone	4.1	59.0	9.6	59.6	3.3	87.6	6.1	88.3	3.3
Progesterone	0.5	63.4	10.7	61.7	10.3	59.8	5.8	70.5	4.3

Compound	LOD <sup>a</sup> (ng L <sup>-1</sup> )	Effluent WWTP3				Effluent WWTP4			
		100 ng L <sup>-1</sup>		500 ng L <sup>-1</sup>		100 ng L <sup>-1</sup>		500 ng L <sup>-1</sup>	
		Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6	Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6	Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6	Recovery (%) n = 6	RSD <sup>b</sup> (%) n = 6
Diethylstilbestrol	13.2	60.0	15.4	58.2	15.0	52.4	18.8	53.2	6.3
17β-estradiol	8.5	—	—	—	—	—	—	—	—
Estrone	4.1	104.2	11.4	121.2	3.7	94.7	10.2	88.9	7.1
Estriol	4.5	54.4	10.1	59.1	15.1	114.1	11.4	89.4	15.7
Norgestrel	1.6	53.9	10.2	48.5	3.8	36.1	13.8	49.1	8.1
Testosterone	1.0	30.2	8.7	47.6	3.3	46.6	12.2	60.9	7.1
Megestrol acet.	1.2	43.8	5.5	58.9	3.9	19.3	14.9	40.3	14.7
Prednisone	9.2	53.1	9.5	61.9	11.4	69.2	11.7	85.7	7.0
Prednisolone	6.1	48.9	5.2	58.7	4.6	69.2	3.5	78.0	8.1
Cortisone	2.1	34.4	4.9	42.7	5.1	48.6	7.0	65.6	3.4
Boldenone	0.7	35.5	4.9	52.5	1.9	40.9	12.8	65.6	5.8
Norethisterone	2.3	22.9	5.3	31.8	5.0	16.7	7.9	49.2	5.1
Nandrolone	4.1	25.3	8.2	41.3	3.4	24.0	4.8	59.2	5.1
Progesterone	0.5	67.7	7.2	79.6	5.9	32.2	14.1	57.0	12.7

<sup>a</sup> Limit of detection. <sup>b</sup> Relative standard deviation.

analysis was performed in gradient mode at a flow rate of 0.3 mL min<sup>-1</sup>. Table 2 shows the gradient used for both the BSM and QSM.

**2.4.2. Mass spectrometry conditions.** The detection and identification of hormones have been developed using an ACQUITY triple quadrupole (TQD) mass spectrometer with an electrospray ionisation (ESI) interface (Waters Chromatography, Barcelona, Spain). All components were controlled using the MassLynx Mass Spectrometry Software. Electrospray ionisation 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 h<sup>-1</sup>. 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

min<sup>-1</sup>. The extractor and RF lens voltages were 3 V and 0.5 V, respectively, in both ionization modes.

The detailed MS/MS detection parameters for each hormone compound are presented in Table 3 and the multiple reaction monitoring (MRM) parameters were optimised by the direct injection of a 1 mg L<sup>-1</sup> standard solution of each analyte into the detector at a flow rate of 10 μL min<sup>-1</sup>.

### 3. Results and discussion

#### 3.1. Optimization of solid-phase extraction (SPE)

**3.1.1. SPE sorbent.** Several authors have reported that lipophilic-hydrophilic balanced polymer and octadecyl carbon chain (C<sub>18</sub>) cartridges and columns have been used to extract endocrine disrupting compounds from waste water samples.<sup>12,20,21,29</sup> Different sample volumes and load and wash

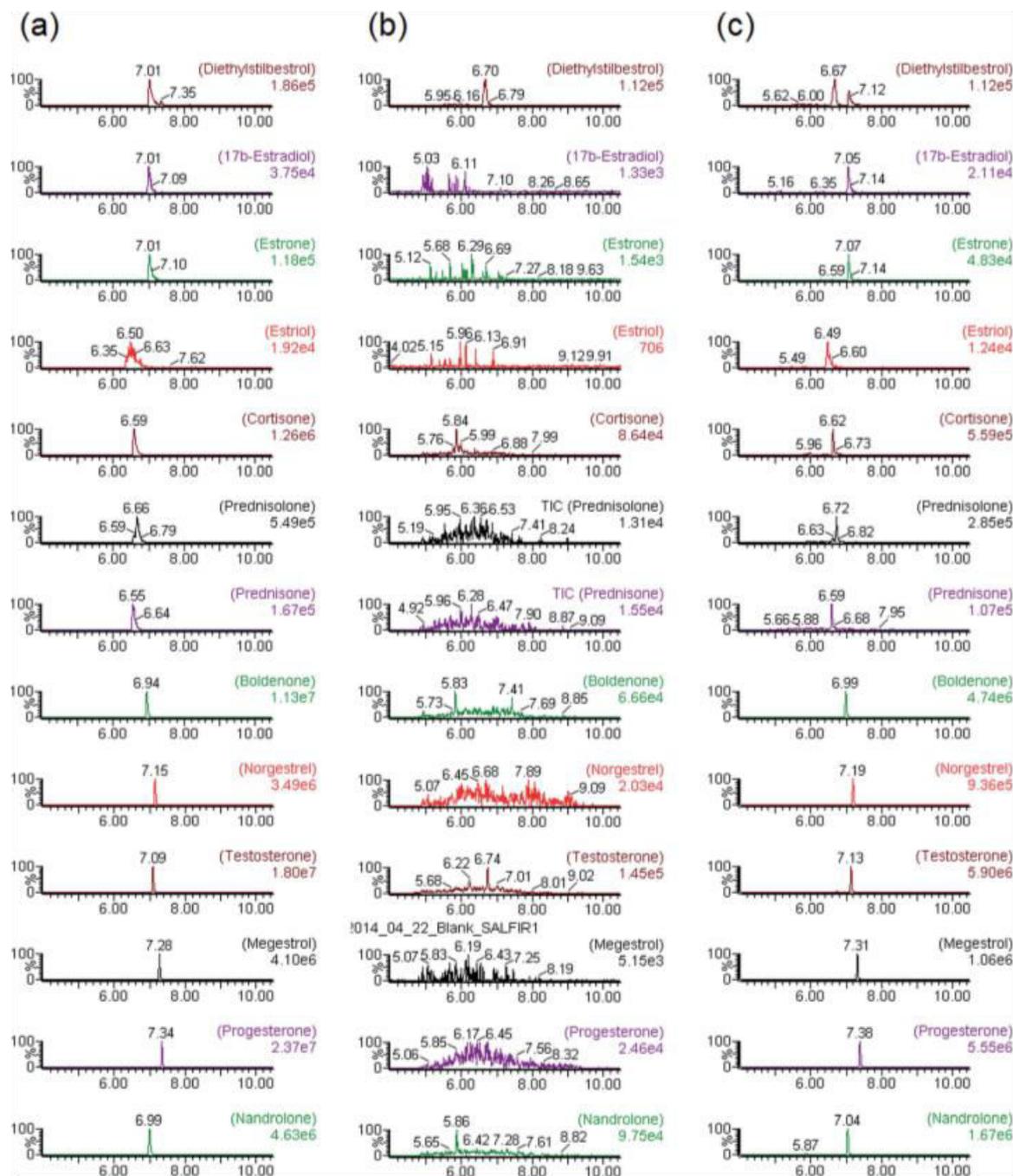


Fig. 3 Chromatograms of the target compounds in pure solvent (a), a non-spiked real sample (b) and a spiked real sample (c).

solvents were tested to achieve higher recoveries and minimize background noise generated by the sample with OASIS HLB and XBridge C<sub>18</sub> SPE columns. To optimize these variables, effluent samples from the tertiary treatment of WWTP1 with a hormone concentration of 500 ng L<sup>-1</sup> were used.

**3.1.2. Sample volume and loading solvent.** The on-line SPE system allows injection sample volumes up to 5 mL in a cycle or several cycles. In our case, we have chosen only one injection cycle because the injection time is 4 minutes as several injection cycles of sample increases the total analysis time. For this

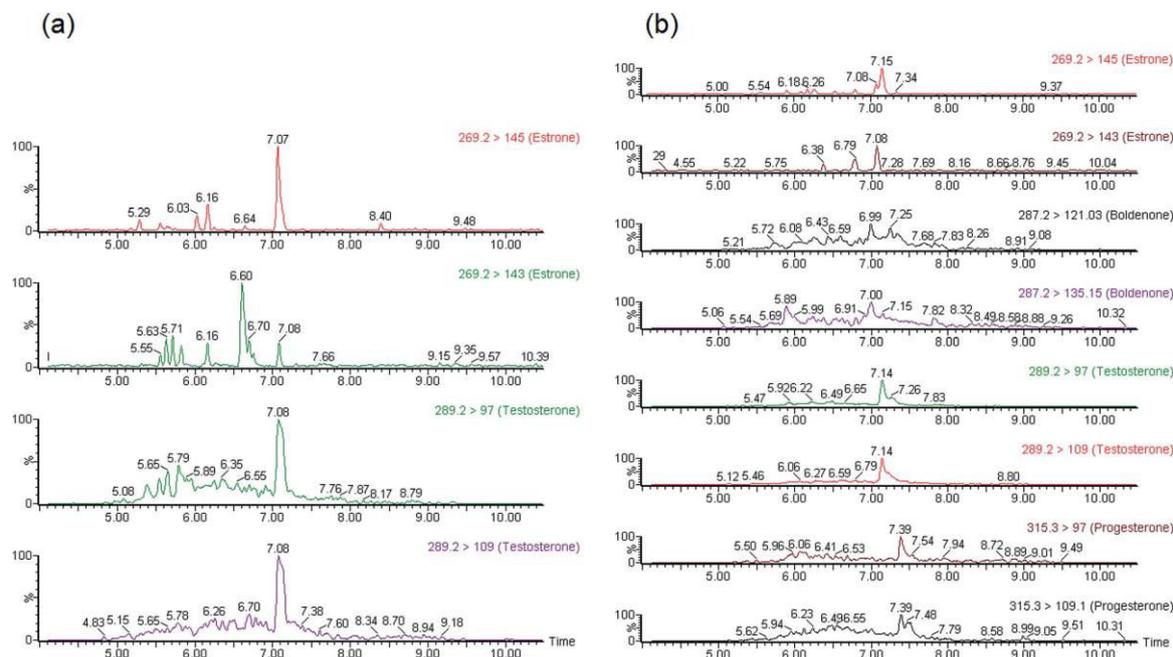


Fig. 4 Chromatograms of the compounds found in the effluent samples from WWTP3 (a) and WWTP4 (b).

reason, volumes from 1 to 5 mL have been tested to check the variations of response area of the analytes.

Another important parameter is the composition of the sample loading solvent because this solvent could improve or diminish the adsorption of analytes onto the SPE column and eliminate interferences from the matrix evaluated. The sample loading solvent is Milli-Q water and four conditions have been evaluated: with 0.1% (v/v) of  $\text{NH}_3$  (pH = 10.1), with 0.03% (v/v) of  $\text{NH}_3$  and 100 mM of ammonium acetate (pH = 8.1), with 0.05% (v/v) of acetic acid (pH = 3.4) and without additives (pH = 5.6).

For both SPE columns tested, maximum recoveries of most compounds were found when the sample volume is between 2 and 3 mL while volumes of 4 and 5 mL showed a significant decrease in recoveries, which may be due to the same sample producing a partial elution of the analytes. For the load phase, the pH between 3.4 and 5.6 showed better recoveries for OASIS HLB SPE columns while, XBridge  $\text{C}_{18}$  showed maximum recoveries at pH = 10.4.

**3.1.3. Sample wash step.** This step is very important because it allows for the elimination of interferences in the sample, thereby providing better signal to noise (*S/N*) ratios. With the on-line SPE system, several combinations and proportions of aqueous and organic solvents can be used in the wash step, automatically, without manipulation by the analyst. We have studied five different proportions of aqueous : organic solvents (100 : 0, 90 : 10, 80 : 20, 70 : 30 and 60 : 40) specifically methanol and water, with and without 0.1% of  $\text{NH}_3$ . Percentages greater than 40% of methanol have not been tested to avoid a co-elution of the analytes under study.

For XBridge  $\text{C}_{18}$  SPE columns, the wash step was eliminated because it caused the elution of the analytes retained in the column. For this reason, the flow rate of the wash step was reduced to the minimum that the UHPLC-MS/MS system allows for (0.01 mL  $\text{min}^{-1}$ ).

For OASIS HLB SPE columns the use of a wash step without  $\text{NH}_3$  produced higher recoveries and better *S/N* ratios for most compounds than the wash step with  $\text{NH}_3$ . An acid wash step has not been tested because it produced the elution of the analytes. Regarding the mixture composition of the wash step, the best results were obtained without adding methanol because the presence of an organic solvent results in deformation of the peaks for most compounds. Fig. 2 shows the peak of nandrolone as an example of this deformation and loss of the *S/N* ratio at different proportions of aqueous : organic solvents used in the wash step.

**3.1.4. pH of the sample.** The pH of the sample is an important parameter because it defines the ionised form of the analytes according to their  $\text{pK}_a$ s. We have tested the acidic pH (pH = 3.5), using acetic acid, the pH of the waste water sample, that was about pH = 5.7 and the basic pH (pH = 10.4) using sodium hydroxide. The results show that the recoveries of several compounds, such as diethylstilbestrol, boldenone and nandrolone were low at pH = 3.5, whereas only two compounds (prednisone and cortisone) had a maximum recovery at this pH using the OASIS HLB SPE column. In contrast, using samples at basic or neutral pH, better recoveries were obtained for most compounds with both types of SPE columns. There were no significant differences between pH = 5.7 and pH = 10.4, but at basic sample pH the recoveries were slightly higher for ten of

Table 5 Concentrations at ng L<sup>-1</sup> of compounds found in effluent samples<sup>a</sup>

Compound	WWTP2	WWTP3	WWTP4
Estrone	nd <sup>b</sup>	<LOQ	14.0 ± 4.9
Testosterone	nd <sup>b</sup>	52.8 ± 1.2	12.6 ± 3.8
Boldenone	nd <sup>b</sup>	nd <sup>b</sup>	5.6 ± 0.3
Progesterone	nd <sup>b</sup>	nd <sup>b</sup>	3.1 ± 0.4

<sup>a</sup> n = 3. <sup>b</sup> Not detected.

the fourteen compounds under study. Using the sample at pH = 10.4, the recoveries were, in all cases, over 63.5% for OASIS HLB SPE columns.

**3.1.5. Desorption step.** Desorption of the analytes from the SPE column was performed using water with 0.1% of NH<sub>3</sub> and methanol, which is the same mobile phase used for chromatographic separation. This desorption step was performed in gradient mode as with the chromatographic separation in the analytical column. Notably, if the analytes can be eluted from the UHPLC column using the solvents in gradient mode, they will be eluted completely from the SPE column, because the retention capabilities are either similar or lower than the retention capabilities of the analytical columns. The use of a gradient mode to elute the analytes is not possible with conventional off-line extraction using SPE cartridges in a manifold. For these reasons, the desorption step in on-line SPE uses smaller volumes of organic solvent and shorter times of extraction, achieving better signal to noise ratios and therefore, lower limits of detection.

Once all the parameters were optimised, we selected the OASIS HLB SPE column, because with this SPE column, the recoveries were higher (over 60% for most compounds) than the recoveries obtained with XBridge C<sub>18</sub> columns (between 9 and 57%).

### 3.2. Analytical parameters and quality control

Linearity, recovery, repeatability, limits of detection and limits of quantification were evaluated for each waste water sample (Table 4) using OASIS HLB SPE columns under the optimum extraction conditions. External calibration curves were prepared from 0.5 to 400 µg L<sup>-1</sup> of each compound. Moreover, three surrogate standards (estrone D2, testosterone D3 and progesterone D9), at a fixed concentration of 200 µg L<sup>-1</sup>, were added to

each calibration level. The linearity was calculated using the relationship between areas and concentrations of compounds and surrogates with excellent correlation coefficients (*r*<sup>2</sup>) higher than 0.990.

The repeatability and recoveries were studied intra-day using six samples of contaminated waste water with hormones at low and high concentration levels (100 and 500 ng L<sup>-1</sup>). These analytical parameters have been studied in samples from the effluent of the tertiary treatment of WWTP1 and in samples from WWTP2, WWTP3 and WWTP4 effluents.

The recoveries calculated are a combination of extraction recoveries and matrix effects on the analytes in the detector due to the impossibility of separating the extraction and identification processes. For most compounds, the recoveries ranged from 50 to 90%, except prednisolone and megestrol acetate that showed recoveries between 120 and 150%, produced by an enhancement of signal from matrix effects. Only diethylstilbestrol and norgestrel presented recoveries below 40%. The waters of WWTP3 and WWTP4 come from a big population and undergo a traditional water treatment, and the recoveries of this waste water were worse than the recoveries of the samples of the other two WWTPs, which work with a membrane bioreactor technology and have a tertiary process to purify the waste water.

The relative standard deviations were satisfactory and similar for most compounds in all samples. At a concentration of 100 ng L<sup>-1</sup> the RSD was slightly to moderately higher than that at a concentration of 500 ng L<sup>-1</sup>. In all cases, the RSDs were lower than 18%.

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 and LOQs ranged from 0.5 to 13.2 ng L<sup>-1</sup> and from 1.66 to 44 ng L<sup>-1</sup>, respectively. These limits are similar to other studies that used off-line SPE with large sample volumes.<sup>19,30,31</sup>

Finally, the method shows a good selectivity as can be seen in Fig. 3. This figure shows the chromatograms of a standard, a non-spiked and a spiked sample.

### 3.3. Analysis of selected compounds in waste water samples

The on-line SPE-UHPLC-MS/MS developed method was applied for the detection of target analytes in different waste water

Table 6 Parameters of other on-line SPE methods used for the determination of steroid hormones in waste water samples

Compounds studied	Sample volume	Analysis time	Average recoveries (%)	Reference
Estrogens	1 mL	13 min	79–95	Salvador <i>et al.</i> <sup>34</sup>
Estrogens progestogens	1 mL	15 min	85–110	Viglino <i>et al.</i> <sup>32</sup>
Estrogens	50 mL	45 min	86–107	Wang <i>et al.</i> <sup>33</sup>
Estrogens, progestogens	10 mL	15 min	71–95	Fayad <i>et al.</i> <sup>28</sup>
Estrogens androgens	50 mL	40 min	31–120	Guo <i>et al.</i> <sup>18</sup>
Estrogens	2.5 mL	10 min	80–98	Ciofi <i>et al.</i> <sup>20</sup>
Estrogens, androgens, progestogens, glucocorticoids	2 mL	15 min	43–95	This study

samples from WWTPs on the island of Gran Canaria (Spain) to check the efficiency of this method. The samples were collected from the effluent of the tertiary treatment of one WWTP and from effluent of three waste water treatment plants that use a membrane bioreactor and activated sludge as treatments. The samples were collected in May of 2014 and January of 2015. To evaluate the matrix effect, three surrogate standards (estrone D2, testosterone D3 and progesterone D9) were added before the extraction process. They could not be added after the on-line extraction due to the configuration of the UHPLC-MS/MS system. To quantify the concentrations of the compounds, the ratios between the peak area of the quantification ions and the peak area of the surrogate standards were used. Fig. 4a and b show the chromatograms of the compounds found in the effluent samples from WWTP3 and WWTP4.

In the effluent of WWTP3, only estrone at a concentration below the quantification limit and testosterone at a concentration of about 50 ng L<sup>-1</sup> were detected. In the effluent sample of WWTP4 four steroid hormones were detected. Progesterone and boldenone were detected at concentrations below 5.6 ng L<sup>-1</sup>, while estrone and testosterone concentrations ranged from 12.6 to 14 ng L<sup>-1</sup>. The concentrations of each compound found are shown in Table 5.

In the effluent samples from WWTP2, any compound under study that was not detected can be interpreted as a removal of the hormone by the treatment used at WWTP. Several authors have stated this removal in different waste water treatment plants all over the world.<sup>13,15,21,32</sup>

Table 6 summarizes the studied compounds, sample volume, analysis time and recoveries obtained in other on-line SPE methods used for the determination of steroid hormones in waste water samples. The whole analysis (extraction and determination) usually takes between 10 and 20 minutes as in the method developed in this paper. However, the studies of some authors, Guo *et al.*<sup>18</sup> and Wang *et al.*<sup>33</sup> present analysis time up to 45 minutes. Another important parameter is the sample volume. In this article, 2 mL of waste water are analyzed, which is a similar volume to that used in other studies by Viglino *et al.*<sup>32</sup> Ciofi *et al.*<sup>20</sup> and Salvador *et al.*<sup>34</sup> These volumes minimize the analysis time as can be seen in Table 6. The recoveries obtained in this article are in the range of the recoveries obtained by other authors. Nevertheless, the main drawback of other on-line SPE methods is the type of steroid hormone that they determine. Ciofi *et al.*, Wang *et al.* and Salvador *et al.*<sup>20,33,34</sup> developed on-line SPE methods only for estrogens, while Guo *et al.*, Fayad *et al.* and Viglino *et al.*<sup>18,28,32</sup> have optimized their methods for estrogens and progestogens or androgens. The on-line SPE method developed in this paper is suitable for estrogens, androgens, progestogens and glucocorticoids. In addition, for the glucocorticoids, this is the first on-line SPE method for their determination, because they have been usually extracted from environmental samples using offline procedures.

## 4. Conclusions

A selective, sensitive and appropriate on-line SPE-UHPLC-MS/MS method for the determination of hormones in waste water

samples at low ng L<sup>-1</sup> concentrations was developed. All of the parameters involved in the extraction step, such as the sorbent type, sample volume, loading solvent, wash solvent and pH of the sample, have been optimized to achieve maximum recoveries. The developed method performs a whole process of extraction, identification and determination of four types of steroid hormones in 15 minutes, is fully automated, and requires only 2 mL of water sample; the parallel work with the samples increases efficiency.

The developed method offers low limits of detection and quantification (LODs and LOQs ranging from 0.5 to 13.2 ng L<sup>-1</sup> and from 1.7 to 44 ng L<sup>-1</sup>, respectively) and high selectivity, which are important in the analysis of these emerging pollutants in environmental and complex matrices. The recoveries have been satisfactory, ranging between 50 and 90% for most compounds in effluent samples, and all of them with RSDs lower than 15% in most cases.

The application of this method to real samples has been satisfactory and four hormones (one oestrogen, two androgens and one progestogen) have been determined in effluent samples with concentrations ranging from 3 to 52 ng L<sup>-1</sup>. No hormones were detected in the effluent sample of the waste water treatment plant that uses membrane bioreactor technology.

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#### **3.1.4. Extracción en fase sólida con polímeros impresos molecularmente acoplada a cromatografía líquida de ultra resolución y detección por fluorescencia para la determinación de estrógenos y sus metabolitos en aguas residuales.**

Las nuevas tendencias en cuanto a selectividad en la extracción de diferentes tipos de contaminantes han promovido el desarrollo de métodos de SPE en los que se utilizan polímeros impresos molecularmente como fase adsorbente para la extracción, lo que se conoce por el acrónimo MISPE (*molecularly imprinted solid phase extraction*). Esta mejora de la extracción en fase sólida permite desarrollar métodos muy selectivos, pues el polímero utilizado como fase adsorbente ha sido diseñado para que extraiga únicamente moléculas de estructura similar a los estrógenos. De esta manera se consiguen extracciones muchos más limpias que con los adsorbentes tradicionales, pues gran cantidad de interferencias no son retenidas en el cartucho de extracción. La unión de estos materiales poliméricos con una técnica tan estandarizada como la SPE permite el desarrollo de métodos reproducibles y perfectamente aplicables como métodos de rutina.

Al igual que ocurre con la SPE tradicional, es necesario optimizar todas las variables que pueden afectar al proceso de extracción. En este caso se ha realizado un diseño experimental para evaluar las condiciones óptimas de variables como el volumen de muestra, el pH de ésta, el paso de lavado o el volumen de disolvente eluyente.

Una vez optimizado, el proceso se comparó con métodos de extracción en fase sólida tradicionales, es decir, utilizando medios

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adsorbentes convencionales. Al realizar esta comparación se pudo comprobar que el método desarrollado con polímeros impresos molecularmente presentaba una extraordinaria selectividad que se tradujo en un cromatograma casi sin ruido de fondo, lo que permite una mejor y más precisa cuantificación de los picos cromatográficos. Asimismo, los límites de detección obtenidos fueron comparables a los obtenidos utilizando medios adsorbentes convencionales, encontrándose éstos entre 0,18 y 0,45 ng·mL<sup>-1</sup>. En lo que se refiere al resto de parámetros de calidad del método, las recuperaciones fueron muy buenas, superiores al 60% y las repetitividads adecuadas, pues las desviaciones estándar relativas fueron inferiores al 10%.

Tras la optimización, la aplicabilidad del método fue demostrada analizando muestras de aguas residuales procedentes de la entrada y salida de una estación depuradora de aguas residuales situada en una zona densamente poblada de la isla de Gran Canaria, por lo que el carácter del agua residual analizada fue eminentemente urbano. Además, se analizaron las aguas residuales de una zona hospitalaria - veterinaria las cuales fueron tratadas mediante un tratamiento primario físico-químico. Dos de las hormonas en estudio (estriol y 17β-estradiol) fueron detectadas en estas aguas, en concentraciones que variaron entre 1,35 y 2,57 ng·ml<sup>-1</sup>. Específicamente, se detectó 17β-estradiol en el influente de la EDAR, mientras que, en los efluentes, tanto en los de la EDAR como en los del hospital veterinario, sólo se detectó un estrógeno, el estriol, en concentraciones que variaron entre 1,35 y 1,63 ng·ml<sup>-1</sup>. La presencia de unos estrógenos a la entrada de la EDAR y otros diferentes a la salida puede explicarse por las relaciones de interconversión que presentan los tres estrógenos naturales.

### *Capítulo 3. Parte experimental y resultados*

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## Research Article

# Molecularly imprinted solid-phase extraction coupled with ultra high performance liquid chromatography and fluorescence detection for the determination of estrogens and their metabolites in wastewater

Estrogens are an important class of endocrine-disrupting compounds, and their contamination of environmental waters through the effluents of wastewater treatment plants could have an important impact on aquatic biota, even at low concentrations. For this reason, the development of selective and sensitive extraction methodologies, which permit the identification and quantification of these compounds at trace level concentrations, is very important. In this study, a quantitative method based on molecularly imprinted solid phase extraction coupled to ultra high performance liquid chromatography with fluorescence detection has been developed. It has been used for the simultaneous determination of three estrogens and two of their metabolites in water samples from wastewater treatment plants. The method developed presents satisfactory limits of detection (between 0.18 and 0.45 ng·mL<sup>-1</sup>), good recoveries (higher than 60%) and low relative standard deviations (under 10%). The method was used to analyze wastewater from a veterinary hospital as well as influent and effluent samples of a wastewater treatment plant of Gran Canaria (Spain) The concentrations of the detected hormones ranged from 1.35 to 2.57 ng·mL<sup>-1</sup>.

**Keywords:** Estrogens / Liquid chromatography / Molecularly imprinted polymers / Solid-phase extraction  
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## 1 Introduction

Emerging pollutants are a wide group of compounds that are continuously increasing because the constant research on them has uncovered the toxicities of different compounds. Among the emerging pollutants, endocrine-disrupting compounds (EDCs), which are a heterogeneous group of chemicals that may affect the endocrine systems of organisms, can be found. Estrogens are female sex hormones that control many body functions such as metabolism, inflammation, and immunological activities, and all of them are considered to be potent EDCs due to their capabilities to produce different disruptions in organisms such as feminization or diminishing reproduction [1, 2]. Both natural and synthetic estrogens

are widely used in human and veterinary medicine as contraceptives and growth promoters and to treat some human cancers [3, 4]. For these reasons, estrogens are now considered to be the most prescribed drugs in the world [5]. Estrogens can suffer several transformations in the body such as hydroxylation, oxidation, and biotransformations producing sulfate and glucuronide metabolites [6], which are also important to study because they can deconjugate into free estrogens in the environment [7].

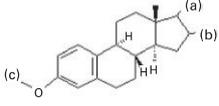
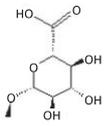
Because a large quantity of estrogens leave the organism through excretions as free estrogens and metabolites, the main emission sources of estrogens into the environment are wastewater treatment plants (WWTPs) [8, 9]. The capacity of WWTP effluents to produce changes in aquatic biota has been known from the early 1990s [10], and in the last few decades, many authors have found the incomplete removal of estrogens from WWTPs [11, 12]; therefore, it is important to study the concentrations of estrogen residues in WWTP effluents to know the impact of these compounds on the environment.

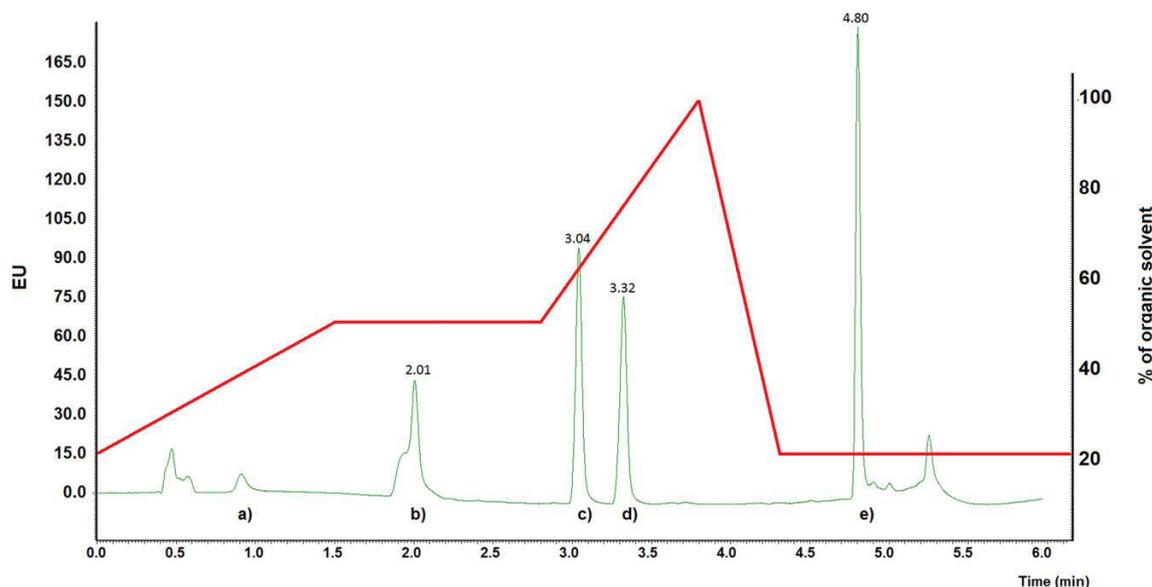
The main challenge in the determination of estrogens in wastewater samples is the development of sensitive extraction and preconcentration methodologies that allow the quantification of this type of compound, which are usually found at very low concentrations. SPE is widely used as a routine technique for the determination of EDCs in environmental water

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**Abbreviations:** DAD, Diode array detector; ECD, Electrochemical detector; EDC, Endocrine-disrupting compound; FD, Fluorescence detection; MDL, Method detection limit; MIP, Molecularly imprinted polymer; MISPE, Molecularly imprinted solid-phase extraction; MQL, Method quantification limit; WWTP, Wastewater treatment plant

**Table 1.** Target compounds under study and their retention times

Estrogens		(a)	(b)	(c)	$t_R$ (min)
	17 $\beta$ -estradiol glucuronide			—H	0.91
	estriol	—OH	·····OH	—H	2.01
	17 $\beta$ -estradiol	—OH		—H	3.04
	17 $\alpha$ -ethynylestradiol	—OH ≡CH		—H	3.32
	17 $\beta$ -estradiol-3-methyl ether	—OH		—CH <sub>3</sub>	4.80



**Figure 1.** Chromatographic separation of the target compounds. In red, the percentage of organic solvent used in the gradient to separate the analytes.

- (A) 17 $\beta$ -estradiol glucuronide
- (B) estriol
- (C) 17 $\beta$ -estradiol
- (D) 17 $\alpha$ -ethynyl-estradiol
- (E) 17 $\beta$ -estradiol-3-methyl ether

samples [13–17]. Nevertheless, with complex samples such as wastewater influents, the extraction efficiency should be lower than for cleaner samples due to the interferences that are retained in the cartridge [16]. Therefore, the use of new sorbents with higher selectivities is necessary.

Molecularly imprinted polymers (MIPs) are synthetic materials that have been processed using molecularly imprinted techniques producing a three-dimensional network that presents a high affinity for a “template” molecule or

structure [18, 19]. This high affinity is appropriate to separate a specific molecule or a family of molecules from environmental samples. Molecularly imprinted polymers have been used as SPE sorbents for the isolation of pharmaceuticals, bisphenol-A, phenolic compounds, or ionic liquids [20–24] from environmental water samples. As well as the high affinity and selectivity of MIPs, they have shown other advantages such as their reproducibilities as well as their great stabilities to different pH values, temperatures, and organic

solvents [25]. Moreover, in molecularly imprinted solid-phase extraction (MISPE) cartridges, stringent washing steps can be applied to remove all interferences, thus minimizing matrix effects and reducing ion-suppression effects.

The aim of this study is the development of a simple procedure for the extraction, preconcentration, and simultaneous determination of three estrogens (estriol, 17 $\beta$ -estradiol, and 17 $\alpha$ -ethinylestradiol) and two metabolites of 17 $\beta$ -estradiol (17 $\beta$ -estradiol-glucuronide and 17 $\beta$ -estradiol-3-methyl ether) in wastewater samples using MISPE followed by UHPLC and fluorescence detection (FD). The optimized method is applied for the identification and quantification of these compounds in wastewater samples obtained from a veterinary hospital as well as the influents and effluents of a wastewater treatment plant (WWTP) of Gran Canaria (Spain).

## 2 Materials and methods

### 2.1 Solvents and reagents

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

MISPE was performed using Affinimip<sup>®</sup> SPE Estrogens from Affinisep (Val-de-Reuil, France). These cartridges have a capacity of 3 mL and 100 mg of sorbent. Classical SPE C<sub>18</sub> cartridges from Waters (Barcelona, Spain) were used. These cartridges were 6 cc Sep-Pak<sup>®</sup> Vac with 500 mg of sorbent.

### 2.2 Sample collection

The samples were collected in a wastewater treatment plant that purifies the water of a high-density population area with a population equivalent of 260 000. Other samples were collected from the effluent of a small chemical-physical treatment plant located in a veterinary hospital. This plant treats the water from the laboratories and other spaces of the veterinary hospital. The samples were collected in February and March of 2015 in 2 L amber glass bottles that were rinsed beforehand with methanol and ultrapure water. At the laboratory, the samples were purified through filtration with fiberglass filters and then with 0.45  $\mu$ m membrane filters (Millipore, Ireland). The samples were stored in the dark at 4°C and extracted within 48 h.

### 2.3 Instrumentation and chromatographic conditions

The instrument used was a UHPLC-FD system consisting of an ACQUITY Quaternary Solvent Manager (QSM) used to load samples as well as to wash and recondition the analytical column, an autosampler with two plates for up to 48 vials, a column manager, and a fluorescence detector with excitation and emission wavelengths of 280 and 310 nm, respectively, which were all from Waters (Barcelona, Spain).

The analytical column was a 50 mm  $\times$  2.1 mm, ACQUITY UPLC BEH Waters C<sub>18</sub> column with a particle size of 1.7  $\mu$ m (Waters, Barcelona, Spain) operating at a temperature of 30°C. The sample volume injected was 10  $\mu$ L, and the analyte separation was carried out using water with 0.1% v/v of ammonia and acetonitrile (ACN) as the mobile phase at a flow rate of 0.3 mL·min<sup>-1</sup> in gradient mode. This gradient started at an 80:20 v/v mixture of water/ACN, which changed to 50:50 v/v in 1.5 min. Then, the gradient remained at 50:50 v/v for 1.3 min and changed to 0:100 v/v in 1 min. Finally, it returned to 80:20 in 0.5 min and stayed at that mixture to stabilize the pressure for an additional 1.7 min. Thus, the chromatographic separation was completed in 6 min. In Fig. 1, a chromatogram of the target compounds at concentrations of 200  $\mu$ g·L<sup>-1</sup> can be seen.

### 2.4 MISPE procedure

To carry out the MISPE, wastewater samples were filtered and spiked with the target compounds at 200  $\mu$ g·L<sup>-1</sup>. The pH of the samples was adjusted with sodium hydroxide and after that, the samples were passed through the MISPE cartridges at a flow of 5 mL·min<sup>-1</sup> at the optimum extraction conditions. The cartridge was washed with 3 mL of ultrapure milli-Q water to eliminate the interferences retained in the sorbent and then, the analytes retained were eluted with 1.5 mL of methanol. After the extraction, to avoid potential carryover effects, the cartridge was washed with methanol and water and dried under vacuum. This clean procedure was checked by injecting the solvents used. The same procedure was used for the analysis of real samples.

## 3 Results and discussion

### 3.1 Optimization of the MISPE

Several parameters can affect the MISPE extraction such as the sample volume, the sample pH, and the elution volume. Although the cartridge used has a protocol (Table 2), we have studied several modifications to this protocol to achieve better recoveries and preconcentration factors. To optimize these parameters, samples of wastewater treatment plant effluent spiked with a solution of the five fluorescent estrogens to obtain a final concentration of 200  $\mu$ g·L<sup>-1</sup> were used.

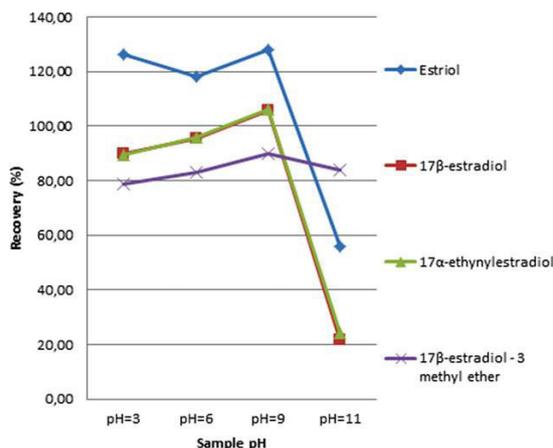
The first studied parameter was the sample volume. Two different volumes (50 and 100 mL) were tested. The volume

**Table 2.** Differences between proposed and optimized protocols of use of AFFINIMIP SPE Estrogens cartridges

Step (flow rate)	Proposed protocol for water samples	Optimized protocol for wastewater samples
Equilibration (2 drops/s)	<ul style="list-style-type: none"> <li>3 mL acetonitrile</li> <li>3 mL ultrapure water</li> <li>Do not allow the cartridge to dry</li> </ul>	<ul style="list-style-type: none"> <li>3 mL acetonitrile</li> <li>3 mL ultrapure water</li> <li>Do not allow the cartridge to dry</li> </ul>
Loading (1 drop every 2 s)	<ul style="list-style-type: none"> <li>Up to 50 mL water (to be analyzed)</li> </ul>	<ul style="list-style-type: none"> <li>50 mL of wastewater at pH = 9</li> </ul>
Washing of interferents (1 drop/s)	<ul style="list-style-type: none"> <li>3 mL ultrapure water</li> <li>3 mL of 60/40 ultrapure water/acetonitrile</li> </ul>	<ul style="list-style-type: none"> <li>3 mL ultrapure water</li> </ul>
Drying	<ul style="list-style-type: none"> <li>Apply vacuum or nitrogen flow through cartridge during 3–5 min</li> </ul>	<ul style="list-style-type: none"> <li>Apply vacuum or nitrogen flow through cartridge during 3–5 min</li> </ul>
Elution (1 drop/s)	<ul style="list-style-type: none"> <li>3 mL Methanol</li> </ul>	<ul style="list-style-type: none"> <li>1.5 mL Methanol</li> </ul>

of 50 mL provided better recoveries for the three estrogens studied, and only for estriol was the recovery using 100 mL of sample slightly higher than for the 50 mL sample volume. No higher volumes were tested to prevent plugging of the cartridge and the co-elution of the analytes with the sample.

Another important parameter is the sample pH. Four different pH values were tested, three pH values (pH = 3, 6, and 9) below the  $pK_a$  values of the compounds and one pH value over the  $pK_a$  values of the compounds (pH = 11). To achieve a pH of 3, acetic acid was used, while sodium hydroxide was used to adjust the pH to 9 and 11. A micro-pH



**Figure 2.** Effect of the sample pH in the extraction of target compounds.

2002 pH-meter from Crison Instruments (Barcelona, Spain) was used to measure the exact pH of each sample and they were immediately extracted. The recoveries for pH values of 3, 6, and 9 were acceptable, ranging from 80 to 125%, while the recoveries for a pH of 11 were moderately lower and below 80%. As seen in Fig. 2, the extractions at pH values lower than the  $pK_a$  values of the compounds are more effective because the extractions are performed with neutral molecules. When the sample pH value is greater than the  $pK_a$  values of the target compounds, the efficiency of the extraction decreases.

The washing step defined in the protocol consists of a first step using ultrapure water and a second step using a mixture of water and acetonitrile. Nevertheless, washing with organic solvent produced a partial elution of the compounds retained in the cartridge, especially the polar compounds with a peak at the beginning of the chromatogram found to be estriol. Therefore, it was determined that only a washing step with ultrapure water should be used.

Finally, the recoveries with two different elution volumes (1.5 and 3 mL) were tested. The recoveries obtained were similar using both volumes; thus, 1.5 mL of organic solvent was chosen because it provides a better preconcentration factor, which was 33.3.

In accordance with the obtained results, the optimum conditions for the MISPE procedure were as follows: 50 mL of sample at a pH of 9, a washing step with ultrapure water, and desorption with 1.5 mL of methanol in one step (Table 2). Moreover, it was observed that the 17β-estradiol-glucuronide was not retained in the MISPE cartridge in any experiment, so this compound was not considered in posterior quantitative studies.

### 3.2 Comparison between MISPE and classical SPE

Once the MISPE extraction was optimized, the results were compared with a  $C_{18}$  method developed in a previous

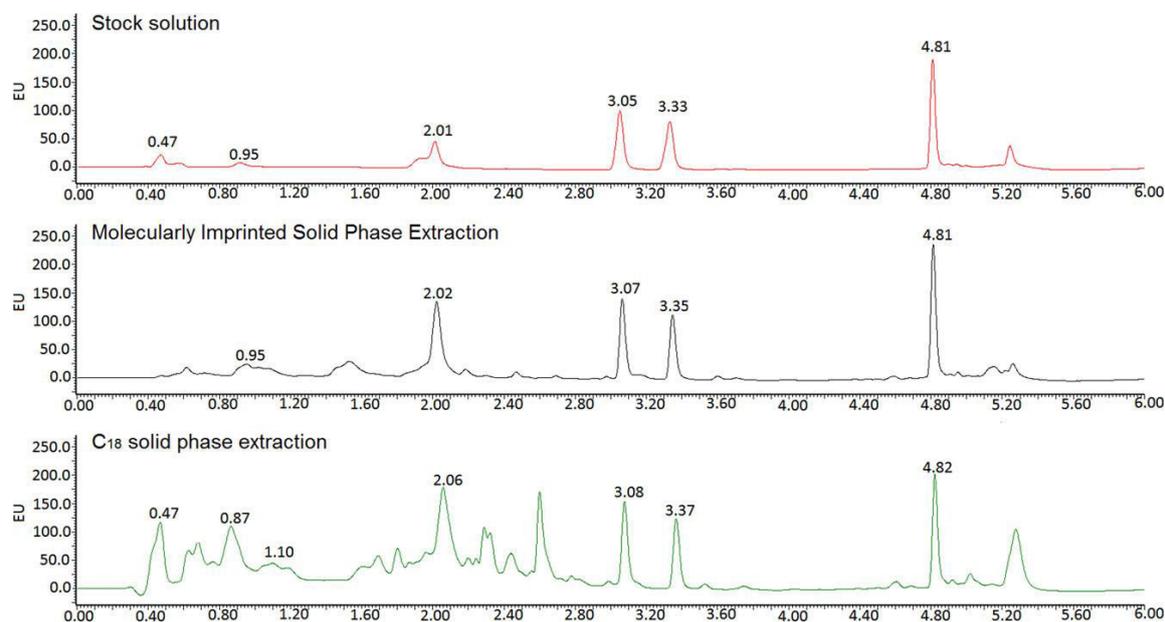


Figure 3. Chromatograms obtained injecting the stock solution, and the extracts of MISPE, and classical SPE.

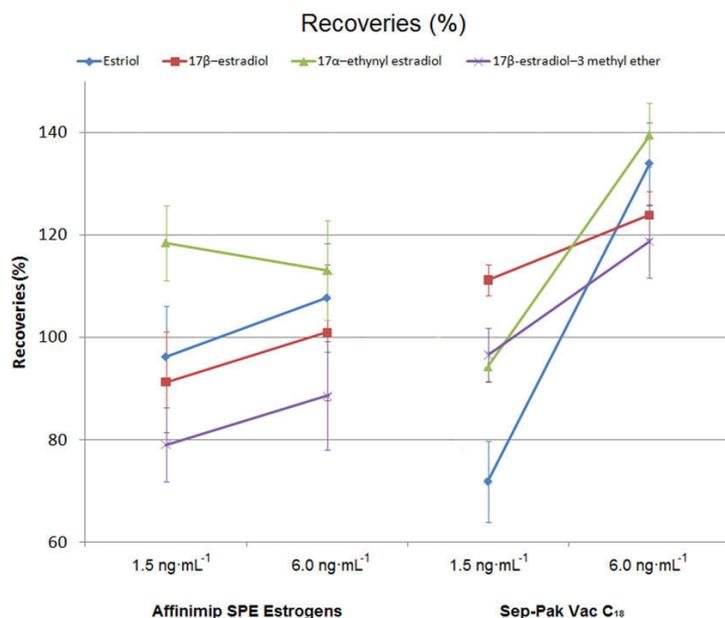


Figure 4. Recoveries obtained using Affinimip SPE (MISPE) Estrogens and Sep-Pak Vac C<sub>18</sub> cartridges at two concentration levels.

article [26]. The classical SPE method was based on a C<sub>18</sub> cartridge and used a sample volume of 250 mL at a pH of 8. Spiked effluent samples with a concentration of 200 μg·L<sup>-1</sup> of the compounds were used to compare both methods. Figure 3 shows the chromatograms of a stock solution in methanol and the extracts of MISPE and classical SPE, with all of them having the same concentration of the analytes (200 ng·mL<sup>-1</sup>). In this figure, it is clear that the chromatogram

obtained with molecularly imprinted polymer cartridges presented less interferences than the chromatogram using non-imprinted polymer cartridges. For this reason, the molecularly imprinted polymer cartridge showed a better selectivity, which is very important in analysis with optical detectors. In addition, the LODs for the target compounds were better using MISPE than classical SPE. Both cartridges did not present significant differences in their recoveries and

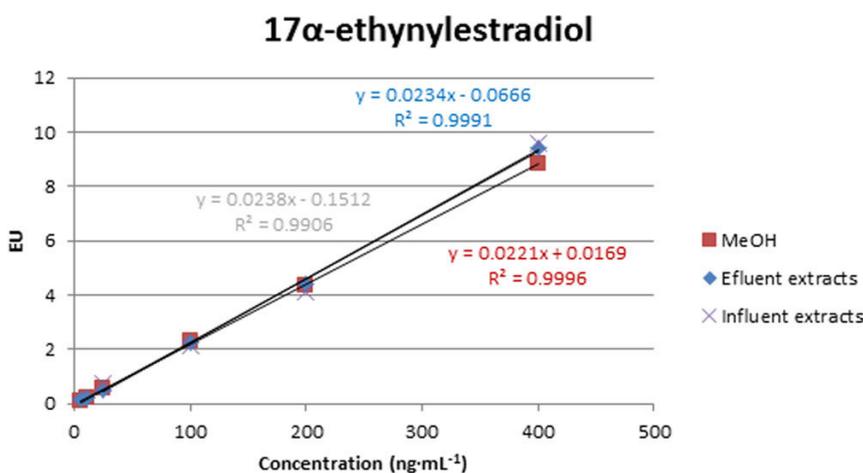
**Table 3.** Analytical parameters of the developed method for the different samples studied

Compound	MDL <sup>a)</sup> (ng·mL <sup>-1</sup> )	MQL <sup>b)</sup> (ng·mL <sup>-1</sup> )	Influent WWTP		Effluent WWTP		Effluent veterinary hospital	
			Recovery (%) <i>n</i> = 6	RSD <sup>c)</sup> (%) <i>n</i> = 6	Recovery (%) <i>n</i> = 6	RSD <sup>c)</sup> (%) <i>n</i> = 6	Recovery (%) <i>n</i> = 6	RSD <sup>c)</sup> (%) <i>n</i> = 6
Estriol	0.41	1.37	40.0	1.8	94.3	4.1	87.0	5.0
17β-estradiol	0.36	1.20	80.7	0.6	62.4	9.7	105.8	5.4
17α-ethynyl estradiol	0.45	1.50	75.8	2.5	60.0	7.1	90.8	3.3
17β-estradiol-3-methyl ether	0.18	0.60	86.1	4.3	66.0	1.7	98.8	3.5

a) Method detection limit.

b) Method quantification limit.

c) RSD.



**Figure 5.** Calibration curves for 17α-ethynylestradiol made in methanol and effluent and influent SPE extracts.

reproducibilities. Their recoveries were between 70 and 120%, and the RSDs were below 10% in all cases. The linearities of both procedures were also studied, and there were no important differences between them. Figure 4 shows the recoveries obtained for both cartridges using effluent wastewater samples spiked with the target compounds at two concentration levels. The recoveries at the high concentration level using the C<sub>18</sub> cartridge are over 110% because the selectivity of this cartridge is worse than that of the molecularly imprinted polymers and more interferents affect the chromatogram.

### 3.3 Analytical parameters

The linearity, recovery, repeatability, LODs, and LOQs were evaluated using molecularly imprinted SPE cartridges at the optimum extraction conditions (Table 3). The analytical parameters were studied in samples from the influent and effluent of a WWTP and in effluent samples from a veterinary hospital. External calibration curves were prepared for each compound from 0.5 to 400 ng·mL<sup>-1</sup>, and there were not important differences between the calibration curves made in

methanol and those made in the different SPE extracts. Figure 5 shows these three calibrations for 17α-ethynylestradiol, and it can be observed that the calibration curves are very similar. The linearity of each curve was evaluated using the correlation coefficient (*r*<sup>2</sup>), which was higher than 0.990 in all cases.

The repeatability and recoveries were studied using six samples of wastewater spiked with the target compounds at a concentration level of 1.5 ng·mL<sup>-1</sup> by evaluating the RSDs. They were similar for most compounds, and in all cases, the RSDs were satisfactory because they were below 10%. The recoveries were evaluated by calculating the ratio between the response of the extracted sample with analytes and the response of postextracted spiked samples [27]. As seen in Table 3, the higher recoveries were obtained in the veterinary hospital effluent samples, and all of them were over 87%. For the WWTP samples, the recoveries were over 60% in all cases, except for estriol in the influent sample, which had a recovery of 40%.

The method detection limit (MDL) and the method quantification limit (MQL) for each compound were calculated from the S/N of each individual peak. The MDL was defined as the lowest concentration that gave an S/N that was greater than 3. The MQL was defined as the lowest

**Table 4.** Comparison of different chromatographic methods based in molecularly imprinted SPE for the determination of estrogens in environmental water samples

Compounds	Matrix studied	Determination technique	Method detection limits	References
Estrone	River water	UHPLC–MS/MS	4.5–9.8 ng·L <sup>-1</sup>	[25]
Estriol	Tap water			
17β-estradiol				
17α-ethinylestradiol				
Estrone	River water	LC–MS/MS	LOQs: 1.9–4.0 ng·L <sup>-1</sup>	[31]
Estriol				
17β-estradiol				
17α-ethinylestradiol				
Estrone	River water	LC–MS/MS	0.7–1.9 ng·L <sup>-1</sup>	[32]
Estriol				
17β-estradiol				
17α-ethinylestradiol				
Estrone	Water	HPLC–DAD	DAD: 8.7–11.0 μg·L <sup>-1</sup>	[30]
Estriol		HPLC–ECD	ECD: 0.071–0.19 μg·L <sup>-1</sup>	
17β-estradiol				
Estrone	Mineral water	HPLC–MS/MS	0.05–0.16 μg·L <sup>-1</sup>	[33]
17β-estradiol				
17α-ethinylestradiol	Wastewater			
Estriol	Wastewater	UHPLC–FD	0.18–0.45 μg·L <sup>-1</sup>	This study
17β-estradiol				
17α-ethinylestradiol				
17β-estradiol-3-methyl ether				

**Table 5.** Concentrations in nanogram per milliliter of target compounds in the samples studied

Compound	WWTP influent	WWTP effluent	Veterinary hospital effluent
Estriol	nd <sup>a)</sup>	1.35	1.63
17β-estradiol	2.57	nd <sup>a)</sup>	nd <sup>a)</sup>
17α-ethynyl estradiol	nd <sup>a)</sup>	nd <sup>a)</sup>	nd <sup>a)</sup>
17β-estradiol-3 methyl ether	nd <sup>a)</sup>	nd <sup>a)</sup>	nd <sup>a)</sup>

a) Not detected.

concentration that gave an S/N that was greater than 10. The MDLs calculated for the target compounds ranged from 0.18 ng·mL<sup>-1</sup> for 17β-estradiol-3 methyl ether to 0.45 ng·mL<sup>-1</sup> for 17α-ethynylestradiol. These limits are slightly better to those found in other studies that determined estrogens by LC or UHPLC with an optical detector and traditional sorbents in SPE [28–30]. In Table 4 it is shown that a comparison with other chromatographic methods based on SPE using imprinted polymers. Lucci et al. and Matějčiček et al. [25, 31, 32] use MS as detection technique and they achieve MDLs in the range of nanogram per liter in clean environmental waters as tap or river waters. Nevertheless, when the sample studied is more complex, like a wastewater, the MDLs are similar to the limits achieved in this work, even using MS as detection technique [33] Moreover, in comparison with other optical detection techniques as DAD [30] the detection limits of the developed method using fluorescence detection are one hundred times lower.

### 3.4 Analysis of estrogens and their metabolites in wastewater samples

The developed method was applied for the determination of target analytes in wastewater samples. The samples were collected from the influent and effluent of a WWTP that treats the water of a large population area using a conventional treatment of activated sludge as well as from the effluent of a veterinary hospital treatment plant.

Only two of the four target compounds being studied were detected in the three studied samples (Table 5). Neither 17α-ethynylestradiol nor 17β-estradiol-3-methyl ether was detected in any of the samples. 17β-estradiol was detected at a concentration of 2.57 ng·mL<sup>-1</sup> only in the influent of the WWTP, while concentrations from 1.5 to 2 ng·mL<sup>-1</sup> of estriol were detected in both effluents. The presence of free estriol in the effluents could be explained by the deconjugation of estrogens made by fecal bacteria once the wastewater reached the WWTP [34].

## 4 Concluding remarks

The developed MISPE–UHLPC–FL method for the determination of estrogens in wastewater samples shows a good selectivity and sensitivity. All of the parameters involved in the extraction step, such as the sample volume, pH of the sample, wash solvent, and elution volume, have been optimized to achieve maximum recoveries.

The developed method is cheaper than chromatographic methods coupled to MS detection and offers low detection limits that ranged from 0.18 to 0.45 ng·mL<sup>-1</sup>, which are appropriate in the analysis of free and conjugated estrogens in environmental and complex matrices. The recoveries have been satisfactory, over 60% for most compounds in all of the samples and all of them with RSDs lower than 10%.

The application of this method to real samples has been satisfactory, and estriol and 17β-estradiol have been determined in wastewater samples with concentrations ranging from 1.4 to 2.5 ng·mL<sup>-1</sup>. Neither 17α-ethynylestradiol nor 17β-estradiol-3-methyl ether were detected in any of the samples.

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The authors have declared no conflict of interest.

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**3.1.5. Determinación de andrógenos y progestágenos en muestras medioambientales y biológicas usando extracción por adsorción sobre dispositivos químicamente modificados acoplada a cromatografía líquida de ultra resolución con espectrometría de masas en tándem**

Este último artículo, en lo referente a análisis de hormonas en muestras líquidas, aborda el desarrollo de una metodología de extracción miniaturizada desarrollada en 2014 por Kabir y colaboradores conocida como extracción por adsorción sobre dispositivos químicamente modificados (FPSE). La técnica está basada en la extracción de los analitos por adsorción sobre un polímero que se encuentra depositado sobre una pequeña porción de un material flexible como la celulosa, la cual actúa como soporte. A pesar de ser una técnica de extracción de equilibrio, presenta la ventaja de que la capacidad extractiva es muy alta, debido a la elevada superficie adsorbente en comparación con otras técnicas de equilibrio como la SBSE o la SPME. La FPSE presenta importantes beneficios, en comparación con otras técnicas de extracción, como sencillez, minimización de volúmenes de muestra y de disolventes extractantes, tiempos cortos de extracción y baja manipulación de la muestra.

En este trabajo se presenta, por primera vez, la optimización de un método basado en extracción por adsorción sobre dispositivos químicamente modificados, para andrógenos y progestágenos presentes tanto en agua como en orina. La metodología desarrollada puede ser aplicada, no sólo en el campo de la química analítica ambiental, sino

### *Capítulo 3. Parte experimental y resultados*

también en la clínica, lo que evidencia la versatilidad y robustez de la FPSE. Las variables optimizadas han sido todas aquellas relacionadas con la extracción como por ejemplo volumen de muestra, tiempos de extracción y desorción, volumen y pH de la muestra o naturaleza y volumen del disolvente eluyente. Para realizar esta optimización se llevó a cabo un diseño factorial en el que se estudiaron las correlaciones de las diferentes variables con la eficiencia del método de extracción, así como la relación existente entre ellas.

Una vez optimizado, se comprobó que el método desarrollado brindaba unos excelentes límites de detección, de entre 1,7 y 264 ng·L<sup>-1</sup>, recuperaciones similares a las obtenidas en métodos de extracción convencionales como la extracción en fase sólida y buena linealidad y repetitividad. Seguidamente, su aplicabilidad fue estudiada analizando muestras de agua de grifo, aguas residuales tratadas con diferentes métodos de depuración, aguas residuales procedentes de una zona hospitalaria y orina. Las aguas residuales procedían de una EDAR con tratamiento tradicional de lodos activos en la que se trataron las aguas procedentes de una zona urbana densamente poblada.

Los resultados obtenidos indicaron la no detección de las hormonas estudiadas en las aguas de grifo ni en las aguas residuales sometidas a un tratamiento terciario de depuración. Por su parte, en el efluente del tratamiento secundario, se detectó testosterona a una concentración de 28,3 ng·L<sup>-1</sup>, así como acetato de megestrol, progesterona y boldenona a concentraciones inferiores a sus límites de cuantificación. Los resultados demostraron que las tecnologías terciarias de depuración fueron efectivas en la eliminación de este tipo de hormonas de las aguas residuales. Por su

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parte en las aguas residuales de la zona hospitalaria se detectó progesterona a una concentración de  $227,3 \text{ ng}\cdot\text{L}^{-1}$ , y acetato de megesterol, boldenona y testosterona también a concentraciones inferiores a los límites de cuantificación. Finalmente, en el caso de las muestras de orina, las concentraciones detectadas variaron entre 1,1 y 3,7  $\mu\text{g}\cdot\text{L}^{-1}$ .

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### 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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Urine

#### 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  $\text{ng L}^{-1}$ . 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  $264 \text{ ng L}^{-1}$ ), 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  $227.3 \text{ ng L}^{-1}$  in water samples and from  $1.1$  to  $3.7 \mu\text{g L}^{-1}$  in urine samples.

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#### 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 mineral-corticoids.

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].

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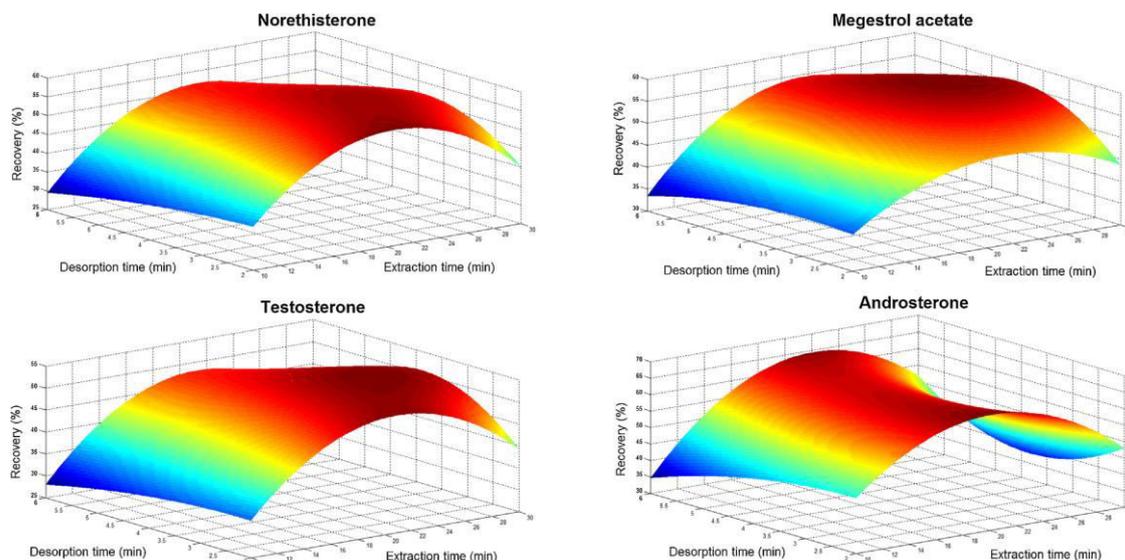


Fig. 1. Response surfaces of  $3^2$  experimental design for the study of the extraction and desorption times.

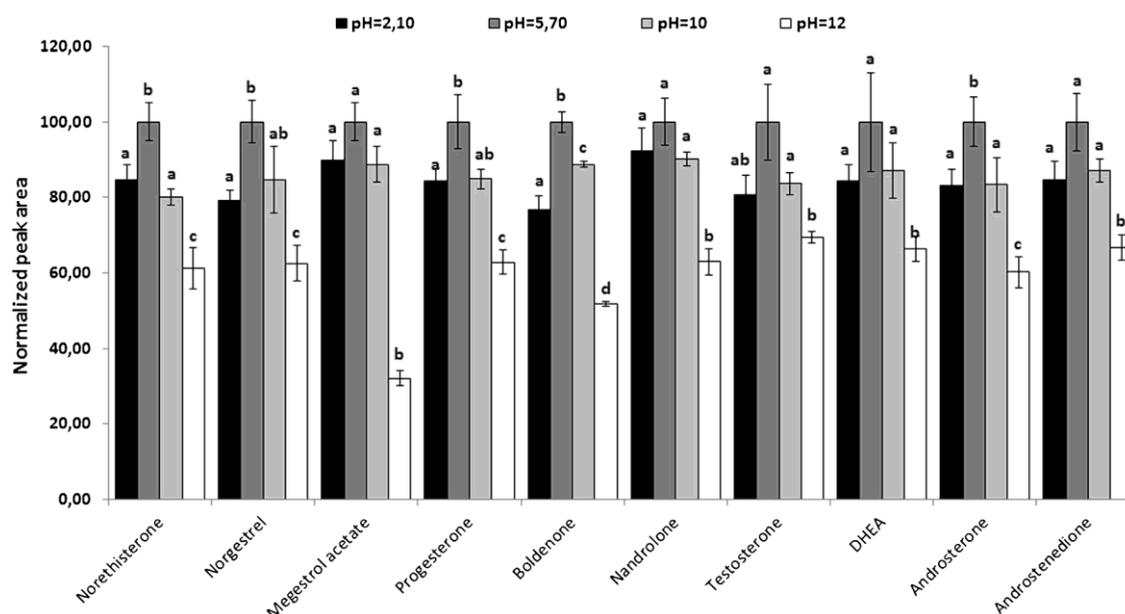


Fig. 2. Effect of sample pH in the extraction efficiency of the FPSE method.

\*Values with the same letter are not statistically different at 5% significance level according to the Dunnnett T3 nonparametric test.

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  $\text{ng L}^{-1}$ ) [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.

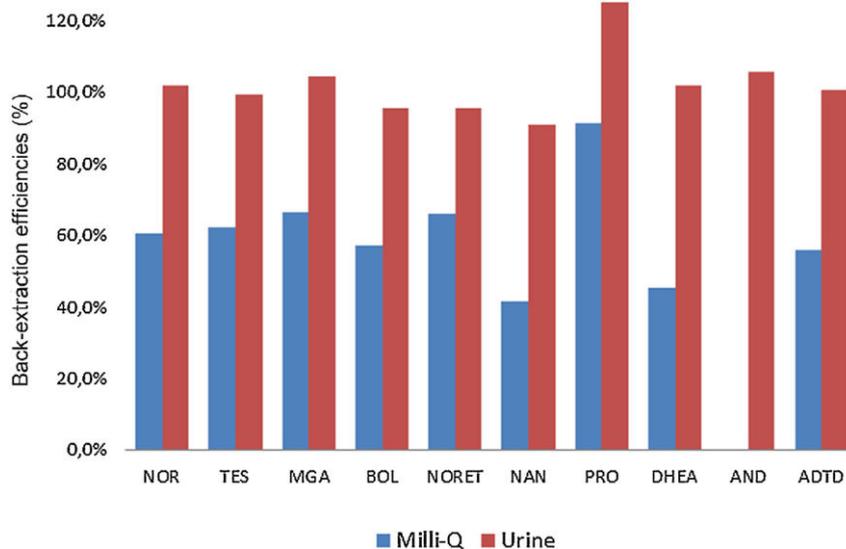


Fig. 3. Back-extraction efficiencies of Milli-Q and urine samples.

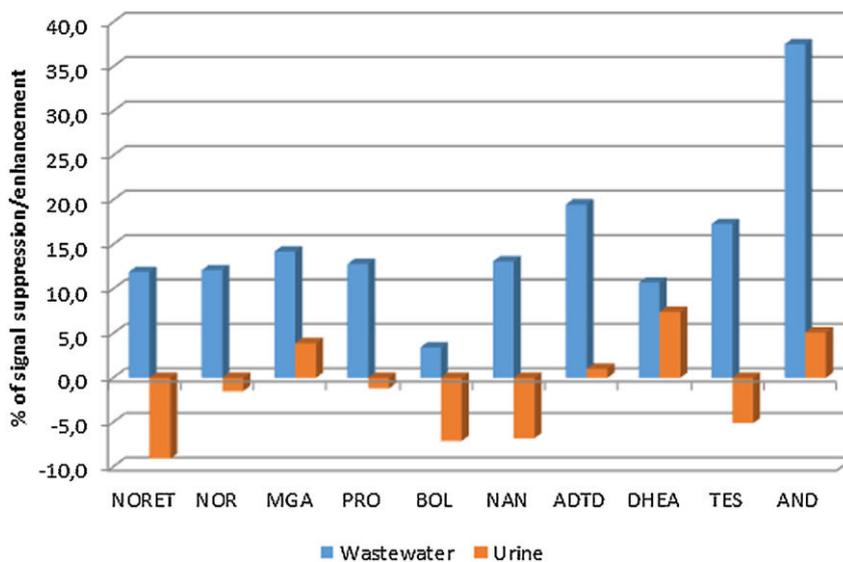


Fig. 4. Analytical signal suppression/enhancement for wastewater and urine samples.

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

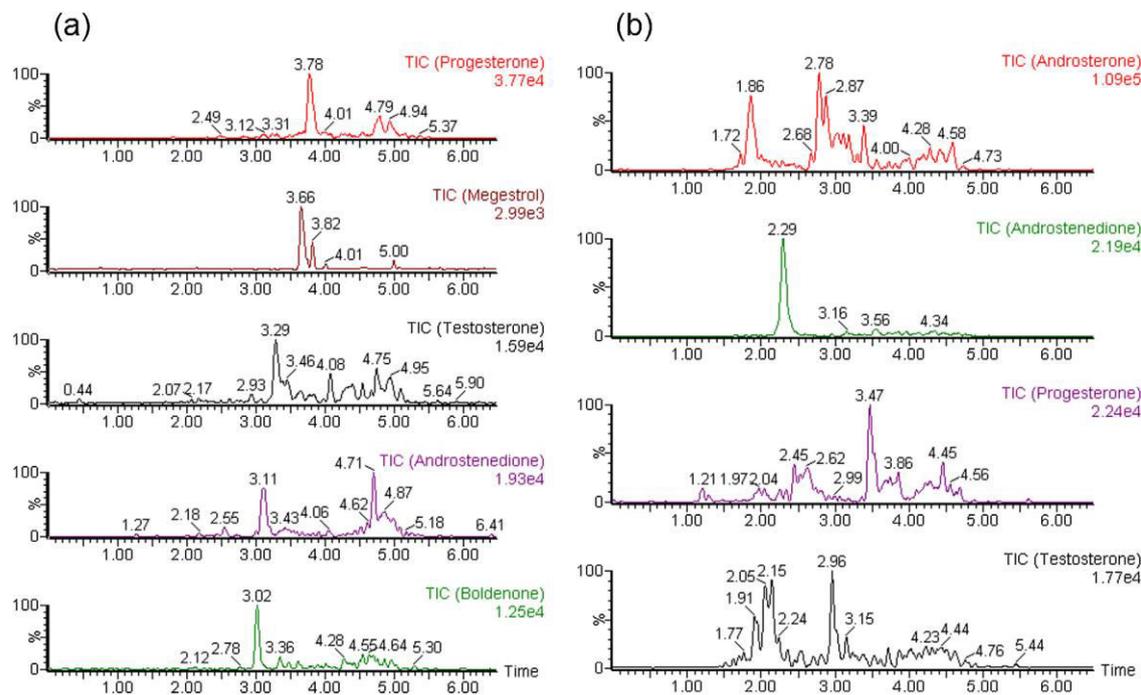


Fig. 5. Chromatograms of (a) hospital untreated wastewater and (b) urine real samples.

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

### 2.1. 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 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  $1000 \text{ mg-L}^{-1}$  of each analyte were prepared by dissolving the compound in methanol, and the solutions were stored in glass-stoppered bottles at  $-20^\circ\text{C}$  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].

### 2.2. Sample collection

#### 2.2.1. 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  $0.22 \mu\text{m}$  membrane filters (Millipore, Ireland), and were stored in the dark at  $4^\circ\text{C}$  and extracted within 48 h.

#### 2.2.2. 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 min and the supernatant was col-

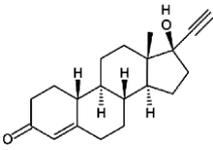
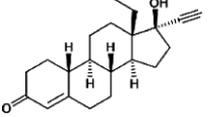
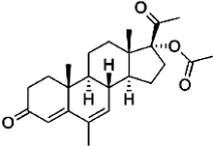
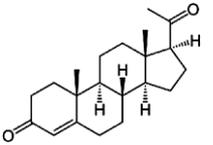
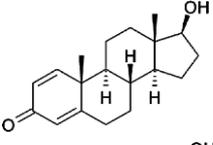
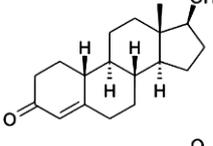
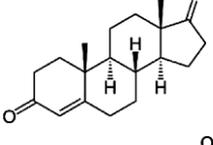
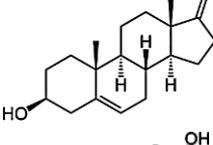
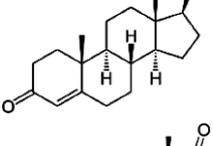
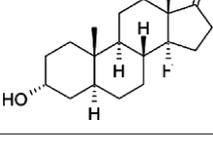
## Capítulo 3. Parte experimental y resultados

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**Table 1**

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

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

<sup>a</sup> From EPI (Estimation Programs Interface) Suite™.

<sup>b</sup> From Scifinder database.

<sup>c</sup> 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 xndesorption 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

lected. 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 min.

### 2.3. 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<sub>18</sub> 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 min<sup>–1</sup> 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 min and to 25:75 (v/v) in 1.25 min more. Then, the gradient changed to 0:100 (v/v) in 1 min. Finally, it returned to 80:20 in 2.25 min and stayed at that mixture for calibration for an additional 0.5 min. Thus, the chromatographic separation was completed in 6.5 min.

### 2.4. 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 min 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 µg L<sup>–1</sup> 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 min and the methanol used for washing was injected to check the absence of target compounds. Finally, the fabrics were dried for 10 min 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<sup>3</sup> fractional factorial experimental design has been used to study the significance of each variable and the correlation/interaction between them. Finally, another 3<sup>2</sup> 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.

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

Compound	LOD <sup>a</sup> (ngL <sup>-1</sup> )	Tap water			Osmosis effluent wastewater			Untreated effluent/biological treated wastewater												
		Relative recovery (%) n=3	Intra-day RSD <sup>b</sup> (%) n=5	Inter-day RSD <sup>b</sup> (%) n=3 × 3	Relative recovery (%) n=3	Intra-day RSD <sup>b</sup> (%) n=3	Inter-day RSD <sup>b</sup> (%) n=3 × 3	Relative recovery (%) n=3	Intra-day RSD <sup>b</sup> (%) n=3	Inter-day RSD <sup>b</sup> (%) n=3 × 3										
NORET	33.5	94.4	6.2	5.2	8.4	6.5	8.4	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	9.5	5.0	9.0	4.4	9.1	94.1	78.5	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	66.6	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	82.7	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	
Urine																				
Compound	LOD <sup>a</sup> (ngL <sup>-1</sup> )	n=3			n=3 × 3															
		Relative recovery (%)	Intra-day RSD <sup>b</sup> (%)	Inter-day RSD <sup>b</sup> (%)	Relative recovery (%)	Intra-day RSD <sup>b</sup> (%)	Inter-day RSD <sup>b</sup> (%)													
NORET	35.2	35.2	1.8	1.8	3.1	3.1	18.0	8.3	8.3	8.3										
NOR	132.3	4.3	4.3	4.2	4.2	4.2	18.1	11.5	11.5	11.5										
MGA	11.1	6.5	6.5	6.5	9.9	9.9	13.0	10.7	10.7	10.7										
PRO	12.8	7.1	7.1	7.1	6.4	6.4	16.9	9.9	9.9	9.9										
BOL	37.9	6.2	6.2	6.2	4.5	4.5	18.8	10.2	10.2	10.2										
NAN	50.1	9.0	9.0	9.0	7.2	7.2	15.6	8.4	8.4	8.4										
TES	8.9	9.3	9.3	9.3	2.8	2.8	14.7	10.1	10.1	10.1										
DHEA	110.6	4.9	4.9	4.9	1.4	1.4	17.2	9.7	9.7	9.7										
AND	80.0	9.2	9.2	9.2	7.9	7.9	9.4	7.8	7.8	7.8										
ADTD	25.6	5.2	5.2	5.2	4.7	4.7	20.0	10.4	10.4	10.4										

<sup>a</sup> Limit of detection.  
<sup>b</sup> 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	Surface waters	Bar adsorptive microextraction	HPLC–DAD	80–100 ng L <sup>-1</sup>	[33]
Norgestrel	Sea water	(BA $\mu$ E)			
Progesterone	Wastewater				
Norgestrel	Surface waters	Automated online	LC–MS/MS	2.5–10 ng L <sup>-1</sup>	[15]
Testosterone	Wastewater	solid-phase extraction			
Androstenedione	Surface water	Solid phase extraction	LC–MS/MS	0.2–2.5 ng L <sup>-1</sup>	[35]
Androsterone	Surface water				
Megestrol acetate	wastewater				
Nandrolone					
Norgestrel					
Progesterone					
Testosterone					
Androstenedione	Wastewater	SPE	GC–MS	1–2 ng L <sup>-1</sup>	[12]
Progesterone					
Testosterone					
Norethisterone	Wastewater	On-line SPE	LC–MS/MS	20–50 ng L <sup>-1</sup>	[17]
Norgestrel					
Progesterone					
Boldenone	Wastewater	On-line SPE	UHPLC–MS/MS	0.5–4 ng L <sup>-1</sup>	[34]
Megestrol acetate					
Nandrolone					
Norethisterone					
Norgestrel					
Progesterone					
Testosterone					
Nandrolone	Wastewater	SBSE	HPLC–DAD	110–180 ng L <sup>-1</sup>	[18]
Progesterone	Seawater				
Testosterone	Surface water				
Androstenedione	Surface water	SPE	LC–MS/MS	0.02–1.44 ng L <sup>-1</sup>	[20]
Androsterone	Wastewater				
Boldenone					
Nandrolone					
Norgestrel					
Progesterone					
Testosterone					
Boldenone	Urine	SPE	LC–MS/MS	170–290 ng L <sup>-1</sup>	[26]
Nandrolone					
Testosterone					
Androstenedione	Tap water	FPSE	UHPLC–MS/MS	2–60 ng L <sup>-1</sup> DHEA: 260 ng L <sup>-1</sup>	This study
Androsterone					
Boldenone					
Dehydroepiandrosterone					
Megestrol acetate					
Nandrolone					
Norethisterone					
Norgestrel					
Progesterone					
Testosterone					

### 3.2. 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<sup>3</sup> 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 min), ionic strength (0 and 15% (w/v) of NaCl) and desorption time (2 and 10 min) 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<sup>2</sup> was built to study the relation between these variables. The levels tested for each parameter were 10, 20 and 30 min of extraction and 2, 4 and 6 min for desorption. In Fig. 1 can be seen response surfaces of the second experimental design for different compounds which show an extraction efficiency maximum in 20 min of extraction and 3 min of desorption.

### 3.3. 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 Fig. 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

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

Compound	WWTP secondary effluent(ng L <sup>-1</sup> )	Hospital untreated wastewater(ng L <sup>-1</sup> )	Urine(μg L <sup>-1</sup> )
Norethisterone	n.d. <sup>a</sup>	n.d. <sup>a</sup>	n.d. <sup>a</sup>
Norgestrel	n.d. <sup>a</sup>	n.d. <sup>a</sup>	<LOQ <sup>b</sup>
Megestrol acetate	< LOQ <sup>b</sup>	<LOQ <sup>b</sup>	n.d.
Progesterone	<LOQ <sup>b</sup>	227.3	1.1
Boldenone	<LOQ <sup>b</sup>	<LOQ <sup>b</sup>	n.d. <sup>a</sup>
Nandrolone	n.d. <sup>a</sup>	n.d. <sup>a</sup>	n.d. <sup>a</sup>
Testosterone	28.3	<LOQ <sup>b</sup>	2.3
DHEA	n.d. <sup>a</sup>	n.d. <sup>a</sup>	n.d. <sup>a</sup>
Androsterone	n.d. <sup>a</sup>	n.d. <sup>a</sup>	<LOQ <sup>b</sup>
Androstenedione	<LOQ <sup>b</sup>	<LOQ <sup>b</sup>	3.5

<sup>a</sup> Not detected.

<sup>b</sup> Value below the quantification limit.

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.

### 3.4. 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. Fig. 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 min 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 min. 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.

### 3.5. 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 μg L<sup>-1</sup> of each compound. Moreover, two internal standards (testosterone D3 and progesterone D9), at a fixed concentration of 200 μg L<sup>-1</sup>, 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*<sup>2</sup>) 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 μg L<sup>-1</sup> and 50 μg L<sup>-1</sup> 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 μg L<sup>-1</sup> and 50 μg L<sup>-1</sup>. 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

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 equal to 3. The LOQ was defined as the lowest concentration that gave a signal to noise ratio that was equal to 10. For environmental waters, the LOD values calculated for the target compounds ranged from 1.7 to 264 ng L<sup>-1</sup>, while they were from 8.9 to 132.2 ng L<sup>-1</sup> for urine samples. The LOQ values were from 5.7 to 880 ng L<sup>-1</sup> for environmental water samples and from 29.7 to 440.7 ng L<sup>-1</sup> for urine samples.

Furthermore, in analysis with MS/MS and electrospray ionization, the composition of complex matrices, as wastewater or urine, has a great influence in the analytical signal. In this sense, an enhancement or suppression of the signal could be produced by co-eluted compounds which would interfere in the good ionization of the compounds under study. To evaluate this phenomenon, spiked matrix extracts and pure standard solutions have been compared in order to evaluate the possible suppressions or enhancements of the analytical signal. Fig. 4 shows that, in wastewater samples, a slightly enhancement of the analytical signal (below 20%) is produced, except for androsterone, which has a signal enhancement of 37.5%. For urine samples, matrix effects have lower values, between -10 and +7% for all the compounds. These low matrix effects show that the developed extraction method has an excellent selectivity and the possible interferences extracted from the samples do not affect the detection of the target analytes.

The FPSE-UHPLC-MS/MS developed method resolves the main drawbacks of other analytical methods for the determination of androgens and progesterogens in environmental samples. As can be seen in Table 5, some authors use other extraction techniques as bar adsorptive microextraction (BAμE) or stir bar sorptive extraction (SBSE) [18,33] with similar sample volumes than the volume used in this work, but the coupling to a chromatographic system with optical detectors causes higher detection limits than the limits reached in this study. Other works use solid phase extraction in

both on-line [15,17,34] and off-line [12,20,35] modes with 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 10–100 times lower than the off-line SPE methods.

### 3.6. 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. Fig. 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  $\mu\text{g L}^{-1}$ . 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  $22.8 \text{ ng L}^{-1}$ . 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-UHPLC–MS/MS method shows a good selectivity and sensitivity and it offers low detection limits that ranged from  $1.7 \text{ ng L}^{-1}$  to  $264 \text{ ng L}^{-1}$ , 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  $\mu\text{g L}^{-1}$ . 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.

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## **3.2. Análisis de muestras sólidas y biológicas**

### **3.2.1. Aplicación de la extracción asistida por microondas y cromatografía líquida de ultra resolución y espectrometría de masas en tándem para el análisis de hormonas sexuales y corticosteroides en lodos de depuradora.**

Tal y como se ha expuesto en la introducción de la presente Tesis Doctoral, el desarrollo de metodologías de determinación de hormonas esteroideas en muestras sólidas está significativamente menos desarrollado que en muestras líquidas. Sin embargo, el estudio de la presencia de hormonas esteroideas en muestras sólidas es vital ya que, teniendo en cuenta los coeficientes de reparto moderadamente altos que presentan las hormonas, es bastante probable que este tipo de contaminantes quede ligado a la fase sólida más que disuelto en la líquida. A pesar de esta importancia, existen pocos métodos de extracción y determinación que permitan el análisis simultáneo de hormonas esteroideas pertenecientes a las diferentes familias en este tipo de matrices.

Teniendo en cuenta lo anterior, en el presente trabajo se presenta una metodología analítica basada en extracción asistida por microondas y separación y determinación por cromatografía líquida de ultra resolución con detección de espectrometría de masas en tándem (MAE-UHPLC-MS/MS) para el análisis de quince hormonas esteroideas. La extracción asistida por microondas permite realizar extracciones eficientes y selectivas, debido a que la energía de las microondas incide directamente en la mezcla de muestra y extractante. Esta selectividad está indicada en

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la extracción de muestras complejas como los lodos procedentes de estaciones depuradoras de aguas residuales, ya que este tipo de muestras presenta una gran cantidad de interferencias que pueden afectar a la extracción y posterior determinación.

En el presente trabajo se optimizaron todas las variables que afectan al método de extracción como composición y volumen del disolvente de extracción, potencia del microondas o tiempo de extracción. Para llevar a cabo este estudio se realizó un diseño experimental que consistía en dos diseños factoriales. En el primero de ellos se evaluaron las correlaciones de cada una de las variables con la eficiencia del método y entre ellas, y en el segundo se estudiaron con mayor profundidad las dos variables que presentaron mayor influencia en el método de extracción. El método optimizado presentó unos límites de detección en el rango de 1,1 a 7,9 ng·g<sup>-1</sup>, idóneos para el análisis de hormonas en muestras de lodos de EDARs. Asimismo, el método mostró una buena reproducibilidad y recuperaciones extraordinarias, superiores al 71% en todos los casos.

Tras la optimización y verificación de la calidad del método, éste fue aplicado a cuatro muestras de lodos procedentes de estaciones depuradoras de aguas residuales que utilizan diferentes métodos de depuración, lodos activos y biorreactor de membrana.

Dos estrógenos, 17β-estradiol y 17α-etinilestradiol, un progestágeno, el levonorgestrel y un glucocorticoide, la cortisona, fueron detectados en las diferentes muestras analizadas en concentraciones que variaron desde 17,3 hasta 1440 ng·g<sup>-1</sup>. En los lodos procedentes de estaciones depuradoras que utilizan lodos activos como tratamiento de depuración, las concentraciones detectadas fueron sustancialmente

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menores que los lodos procedentes de la EDAR basada en biorreactor de membrana. Esto demuestra que el carácter hidrófobo de los lodos obtenidos en el biorreactor de membrana incrementa la capacidad de adsorción de éstos.

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## 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 the 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 15 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 g<sup>-1</sup> and quantification limits which ranged from 3.7 to 26.3 ng g<sup>-1</sup>. 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 × 10<sup>3</sup> ng g<sup>-1</sup>. 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

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### 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 subfamilies 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 comprising 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 treatment plants (WWTPs). The removal of these compounds is mainly done by biodegradation and adsorption on sludge [6–9]; but, in most cases, the compounds 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 subfamily of steroid hormones in the sludge or sediment [16–18].

The main challenge for 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, owing to its simplicity, was ultrasound-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 thermally stable compounds) from environmental and edible matrices [24]. MAE has many advantages, such as the use of small quantities of sample and organic solvents, shorter extraction 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 was 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, were optimized following a statistical experimental design. Once optimized, the method was 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.

## Materials and methods

### 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 L<sup>-1</sup> 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.

### 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 WWTP2 treat the wastewater of two high density areas of the island with equivalent populations of 260,000 and 130,000, respectively. The sludge of WWTP3 and WWTP4 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. WWTP1, WWTP2 and WWTP3 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 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 2015 and January 2016. After the sludge samples were collected, 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 extracted within 48 h.

### 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

**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	17 $\beta$ -Estradiol		2.69
	EE	17 $\alpha$ -Ethinylestradiol		2.70
	E1	Estrone		2.71
Progestogens	DES	Diethylstilbestrol	Progesterone D9	2.74
	NORET	Norethisterone		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

with a 6 EVAP rotor and 6 MF100 vessels (Anton Paar, Graz, Austria).

### Chromatographic and detection conditions

The chromatographic separation was performed using an ACQUITY UPLC BEH Waters C<sub>18</sub> analytical column (50 mm × 2.1 mm, 1.7  $\mu$ m particle size) from Waters (Barcelona, Spain) operated at a temperature of 30 °C. The injected sample volume was 10  $\mu$ L, 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 min<sup>-1</sup> 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 min and then to 25:75 (v/v) in 1.25 min. Then, the gradient changed to 0:100 (v/v) in 1 min. Finally, the mixture returned to 80:20 in 2.25 min and remained at that composition for pressure equilibration for an additional 0.5 min. Thus, the chromatographic separation was completed in 6.5 min.

For the quantitative analysis, the multiple reaction monitoring (MRM) parameters were optimized for each compound. The precursor ions for the oestrogens were [M-H]<sup>-</sup> in negative ion mode (ESI<sup>-</sup>) and were [M+H]<sup>+</sup> in positive ion mode (ESI<sup>+</sup>) 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 L<sup>-1</sup> in methanol was directly infused in the mass spectrometer at a flow rate of 10  $\mu$ L min<sup>-1</sup>, 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 h<sup>-1</sup>. 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 min<sup>-1</sup>. The extractor and RF lens voltages were 3 V and 0.5 V, respectively, in both ionization modes.

In Fig. 1, total ion current (TIC) chromatograms of the target compounds and internal standards at a concentration of 200  $\mu$ g L<sup>-1</sup> are shown.

### 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 min 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 min using the microwave fan and were then left at room temperature for another 10 min. Next, the extracts were filtered using 0.20- $\mu$ m syringe polyethylene terephthalate (PET) filters from Macherey-Nagel (Düren, Germany). The filtered extracts were evaporated

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

Compound	Precursor ion ( <i>m/z</i> )	Cone voltage (ion mode)	Quantification ion, <i>m/z</i> (collision energy, V)	Confirmation ion, <i>m/z</i> (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

Compound abbreviations are defined in Table 1

under a nitrogen stream and were reconstituted with 1 mL of methanol.

## Results and discussion

### Optimization of 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<sup>3</sup> factorial experimental design (three variables: power, irradiation time and extractant volume, at two different levels) was used to study the significance of each variable and the correlation/interaction between them. Then, another 3<sup>2</sup> 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 µg g<sup>-1</sup>) was used.

#### 2<sup>3</sup> 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 min) 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 constructed for each compound and, as can be observed in Fig. 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<sup>2</sup> 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 min for the extraction times. As in the previous experimental design, the experiments were randomized, and the whole design comprised 11 runs because the central point of the statistical grid was analysed three times. No higher

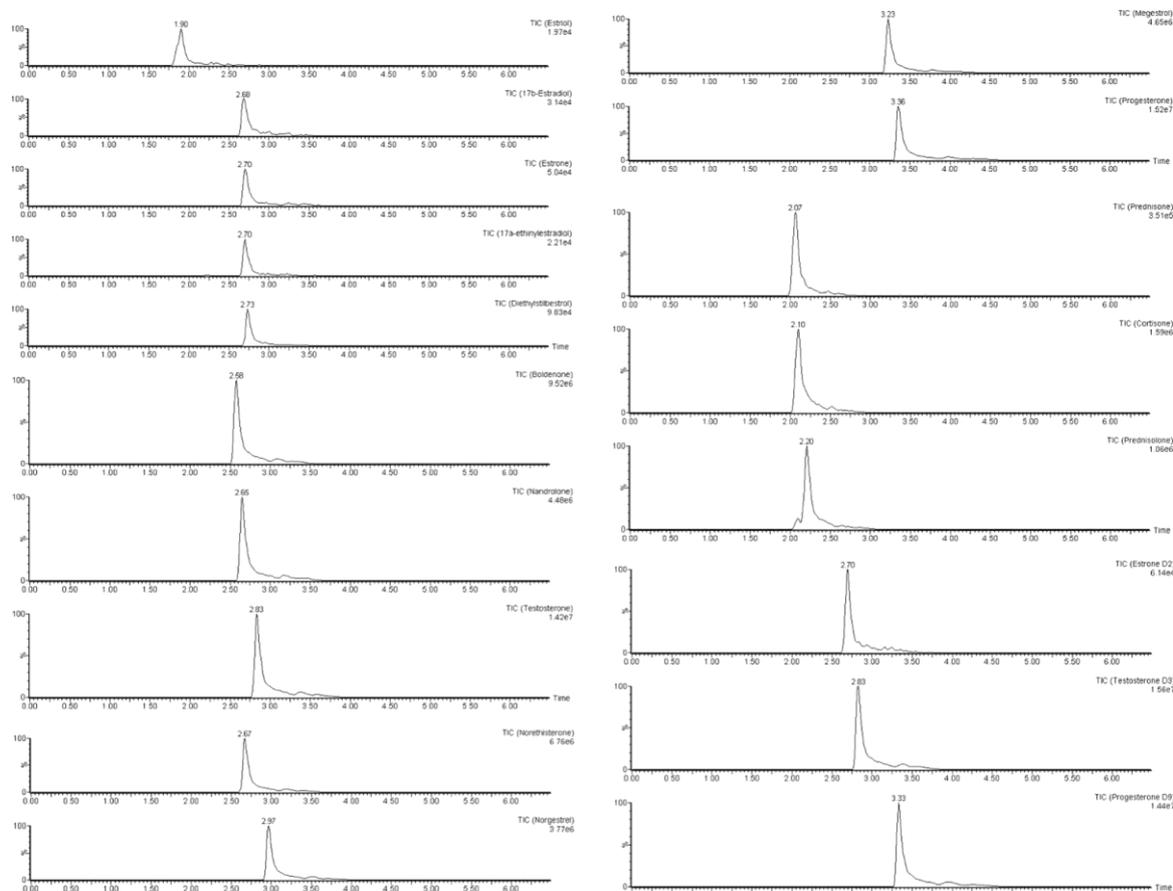


Fig. 1 Chromatograms of the compounds under study at a concentration of  $200 \mu\text{g L}^{-1}$

power or times were considered because at 500 W for 10 min, the extractant solvent was almost evaporated and the accuracy of the method decreased significantly. Using the results, we constructed response surfaces in Minitab 17 for each compound. As seen in Fig. 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 min of extraction using an extractant volume of 10 mL of methanol.

**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 a nitrogen stream 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  $250 \mu\text{g L}^{-1}$ ) before and after the evaporation, and the ratio of 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

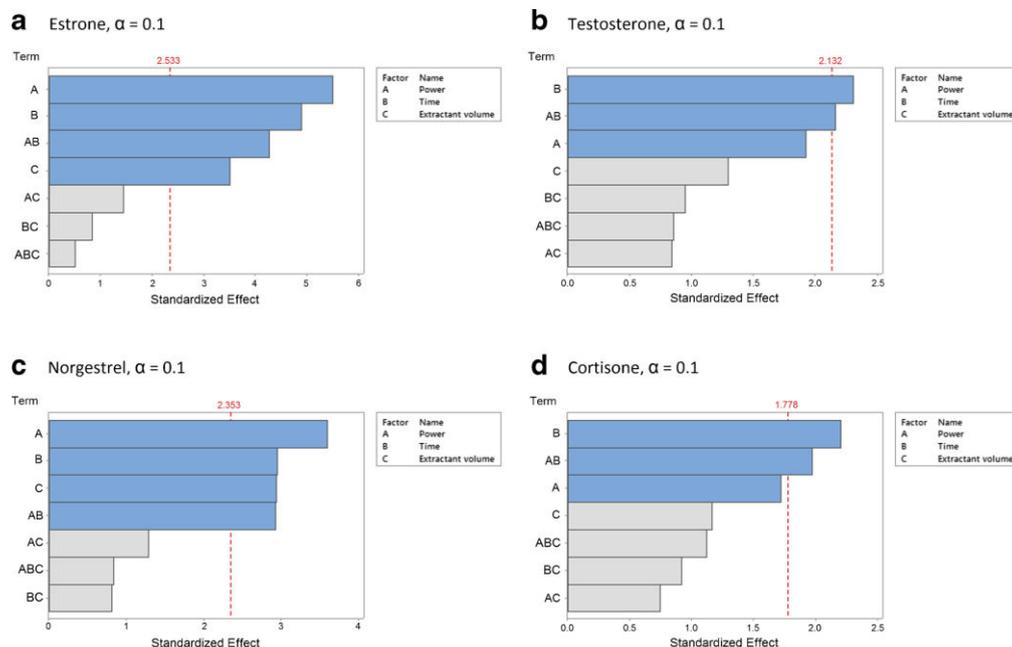


Fig. 2 Pareto charts of the standardized effects for a compound of each hormone subfamily under study

compounds. To evaluate the signal suppression caused by the matrix effects, Vieno et al.'s algorithm [25] was used (Eq. (1)):

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

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  $\mu\text{g L}^{-1}$ ).

The signal suppression was moderate for most of the compounds because the different interferences hindered the ionization of the target hormones because of 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 ion 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  $\mu\text{g L}^{-1}$ . To overcome the different matrix effects of the samples, three internal standards were added to

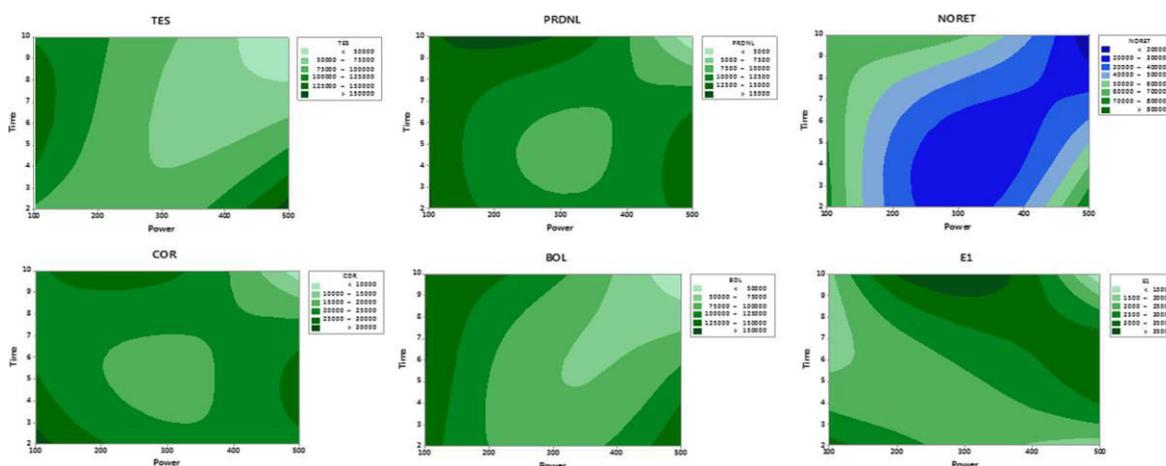


Fig. 3 Response surfaces of the 3<sup>2</sup> factorial design for testosterone, prednisolone, norethisterone, cortisone, boldenone and oestrone

**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 <sup>-1</sup>		250 ng g <sup>-1</sup>		2500 ng g <sup>-1</sup>	
	Evaporation losses	Ion suppression	Evaporation losses	Ion suppression	Evaporation losses	Ion suppression
E3	NA	-52.2	-2.12	46.7	-27.3	12.8
E2	NA	NA	-7.56	37.9	-29.0	-85.1
EE	NA	NA	29.9	3.77	-36.4	-83.1
E1	-31.8	-61.4	9.44	-14.1	-28.0	-79.9
DES	-21.7	-65.2	21.0	26.2	-38.9	-76.3
NORET	-19.6	-68.4	-8.55	-44.2	-3.11	122 <sup>c</sup>
NOR	-13.3	NA	-1.97	NA	-23.2	-65.0
MGA	-37.7	-91.6	-3.23	-83.8	-28.9	-57.8
PRO	6.52	-90.8	-21.8	-81.0	-31.4	-59.2
BOL	7.96	-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	-22.9	3.94	-1.02	-36.0	-45.2
COR	6.81	-39.8	-9.99	3.45	-34.4	-52.2
PRDNL	-31.48	-46.0	-23.4	2.93	-30.4	-55.8

A positive number indicates an enhancement of the analytical signal. Compound abbreviations are defined in Table 1

NA not available

the extracts obtained in the MAE at a concentration of 200 µg L<sup>-1</sup>. For the analysis of oestrogens, a C<sub>18</sub> carbon structure of deuterated oestrogen (oestrone D2) was used as the internal standard, whereas testosterone D3 (C<sub>19</sub> structure) was used for the analysis of androgens and progesterone D9 (C<sub>21</sub> structure) was used for the analysis of progestogens and glucocorticoids.

**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 µg L<sup>-1</sup> of each compound. Moreover, three internal standards (oestrone D2, testosterone D3 and progesterone D9), at a fixed concentration of 200 µg L<sup>-1</sup>, 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*<sup>2</sup>) higher than 0.995 both in external calibration curves and spiked sludge samples.

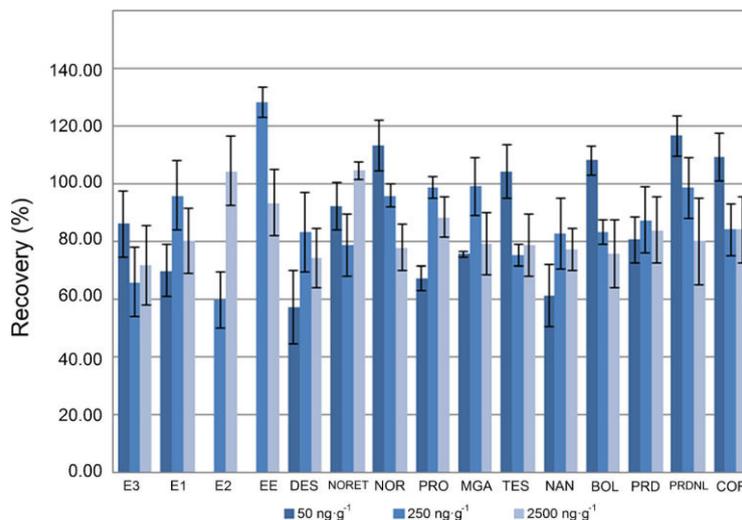
The recoveries of the method were studied by comparing six extracts of spiked sludge samples with six non-spiked sludge extracts spiked after the microwave extraction. This comparison was performed at three concentration levels (50,

250 and 2500 ng g<sup>-1</sup>), and the results in Fig. 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 interday repeatabilities of the method were 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, respectively. The detection limits were from 1.1 to 7.9 ng g<sup>-1</sup>, and the quantification limits ranged from 3.7 to 26.3 ng g<sup>-1</sup>. 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-up step based on solid-phase extraction and required a large quantity of sludge (3 g 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 nine glucocorticoids in sludge samples. The limits of detection

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



were slightly lower than the limits reached in this paper (0.5–1 ng g<sup>-1</sup>), 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 UAE for the extraction of 28 steroids from the four subfamilies of steroids, and the limits were slightly lower than the limits of this study. However, the quantity of sludge used was five times

higher than that used in this study. Finally, the developed method does not require complex sample pretreatments as derivatization [26], 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

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

Compound	LOD (ng L <sup>-1</sup> )	MDL (ng g <sup>-1</sup> )	MQL (ng g <sup>-1</sup> )	Intra-day RSD (%) n = 6			Inter-day RSD (%) n = 3 × 6		
				50 ng g <sup>-1</sup>	250 ng g <sup>-1</sup>	2500 ng g <sup>-1</sup>	50 ng g <sup>-1</sup>	250 ng g <sup>-1</sup>	2500 ng g <sup>-1</sup>
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	NA	18.3	13.5	NA	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	NA
NOR	15.5	2.75	9.18	2.8	4.2	17.5	10.6	NA	NA
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

Compound abbreviations are defined in Table 1

LOD instrumental detection limit, MDL method detection limit, MQL method quantification limit, RSD relative standard deviation, NA 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 g <sup>-1</sup> )	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

Compound abbreviations are defined in Table 1

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 analyses are greener and conducted in shorter times.

**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 because WWTP1 and WWTP2 treat the water of big population areas (more than 100,000 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 bioreactor system. As seen in Table 6 and Fig. 5, only four compounds under study were detected in the different WWTP sludge samples: two oestrogens, 17 $\alpha$ -ethynylestradiol and

17 $\beta$ -estradiol; one progestogen, norgestrel and one glucocorticoid, cortisone.

The sizes of the WWTPs and, subsequently, the volume of treated waters were not correlated 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 the oestrogens, the highest detected concentrations were of 17 $\alpha$ -ethynylestradiol, which ranged from 31.5 to 1.44  $\times 10^3$  ng g<sup>-1</sup>. The other detected oestrogen, 17 $\beta$ -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 in accordance with the affinity adsorption scale determined by Ying et al. [27], which establishes that the adsorption decreases in the order EE > E2 > E1 > E3. Ren et al. [28] concluded that this adsorption change in activated sludge, however, 17 $\alpha$ -ethynylestradiol has a high affinity of adsorption on sludge. Furthermore, the hydrophobic properties of membrane bioreactor sludge produce a better sorption [9] which could explain why the highest concentrations of 17 $\alpha$ -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 an oral contraceptive in combination with 17 $\alpha$ -ethynylestradiol. Moreover, the  $K_{OW}$  value ( $K_{OW} = 3.48$ ) of norgestrel is similar to that of 17 $\alpha$ -ethynylestradiol and 17 $\beta$ -estradiol [29, 30], 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  $\times 10^3$  ng g<sup>-1</sup> and, as happened with 17 $\alpha$ -ethynylestradiol, the highest concentrations were detected in WWTP4. Finally, cortisone was only detected in the sludge samples from the membrane bioreactor wastewater treatment plant (WWTP4) at a concentration of 17.3 ng g<sup>-1</sup>.

**Table 6** Concentrations (ng g<sup>-1</sup>) detected in sludge samples (n = 6) from the four WWTPs under study

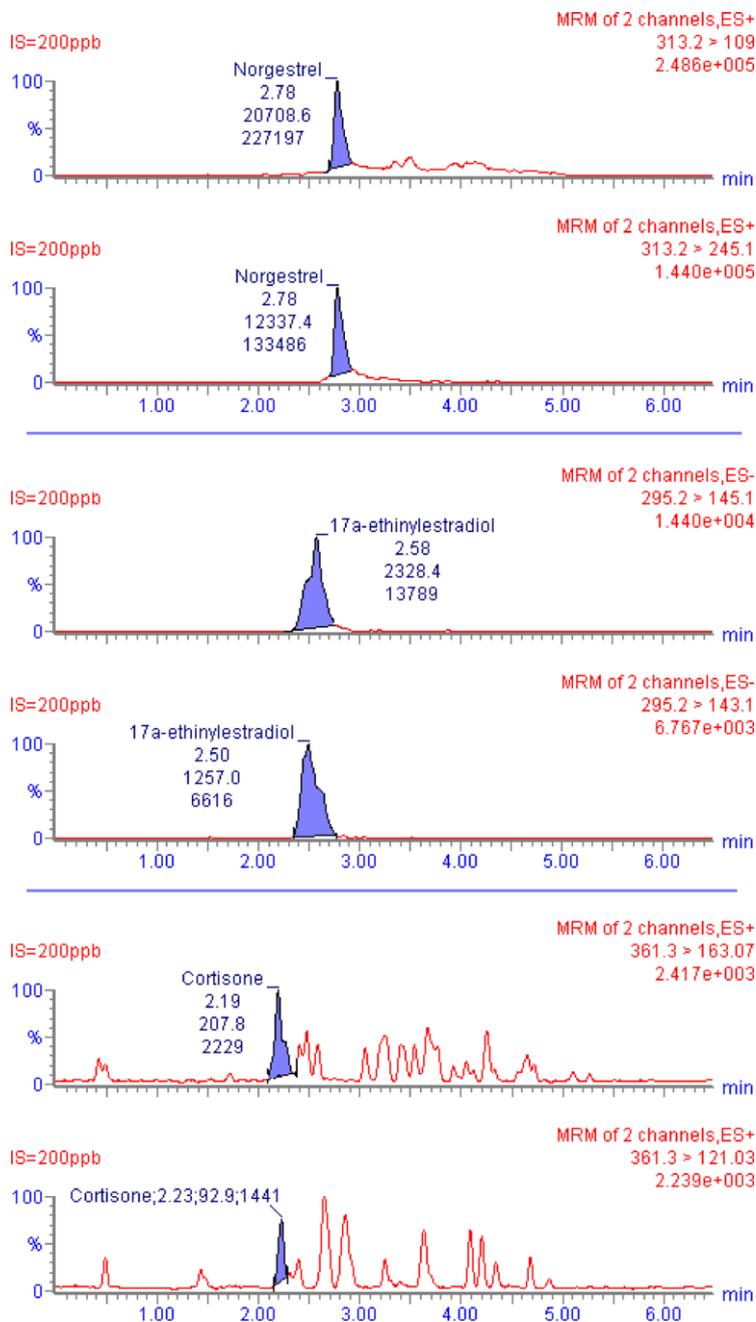
	EE	E2	NOR	COR
WWTP1	31.5 $\pm$ 7.10	<LOQ <sup>a</sup>	874 $\pm$ 66.7	ND
WWTP2	315 $\pm$ 15.7	<LOQ <sup>a</sup>	430 $\pm$ 52.8	ND
WWTP3	ND	<LOQ <sup>a</sup>	981 $\pm$ 65.3	ND
WWTP4	1.44 $\times 10^3 \pm 114$	<LOQ <sup>a</sup>	1.35 $\times 10^3 \pm 74.4$	17.3 $\pm$ 4.27

Compound abbreviations are defined in Table 1

ND not detected

<sup>a</sup> Below quantification limit

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



**Conclusions**

A microwave-assisted extraction combined with ultra-high performance liquid chromatography–tandem mass spectrometry method for the determination of sex hormones and corticosteroids was presented for the first time. In this study, the

MAE-UHPLC-MS/MS method was optimized and successfully applied to sludge samples for the determination of a group of 15 steroid hormones.

The developed method has good selectivity and sensitivity and presents low detection limits that range from 1.11 to 7.90 ng g<sup>-1</sup>, 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 %.

To the best of our knowledge, the developed method is the first one based on microwave-assisted extraction for the determination of steroid hormones from the four subfamilies. 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  $1.44 \times 10^3$  ng g<sup>-1</sup>. The highest concentrations detected belonged to 17 $\alpha$ -ethynylestradiol and norgestrel which are combined and usually used as oral contraceptives. The concentrations of the studied compounds in WWTP4, which uses a membrane bioreactor 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 bioreactor sludge could produce a better sorption of these hormones and thus higher concentrations of hormones in the sludge.

**Acknowledgments** This work was supported by funds provided by the Spanish Ministry of Economy and Competitiveness, Research Project CTM2015-66095-C2-1-R. Rayco Guedes-Alonso thanks the University of Las Palmas de Gran Canaria (Spain) for his Ph.D. student grant. The authors would also like to thank Emalsa S.A., Acciona S.A. and Consejo Insular de Aguas de Gran Canaria for allowing the sampling of sludge at the different wastewater treatment plants.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no competing interests.

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#### **3.2.2. Determinación de hormonas sexuales y glucocorticoides en tejidos de peces usando extracción asistida por microondas y extracción en fase sólida, acopladas a cromatografía líquida de ultra resolución con espectrometría de masas en tándem.**

La preocupación acerca de la presencia de las hormonas esteroideas como compuestos disruptores endocrinos ha crecido en las últimas décadas, por ello es importante el desarrollo de metodologías que permitan su determinación no sólo en aguas o sólidos de ecosistemas contaminados, sino también en los organismos que los habitan. Debido al carácter lipófilo de las hormonas esteroideas, es muy probable que éstas puedan enlazarse a los tejidos de los organismos vivos si éstos se encuentran expuestos. Por todo ello, el conocimiento de los niveles de hormonas de organismos acuáticos permite utilizarlos como bioindicadores del nivel de contaminación sufrido por un determinado ecosistema, pero también es vital conocer estas concentraciones puesto que los organismos acuáticos son parte indispensable de la cadena trófica.

Como se ha podido comprobar en el apartado anterior, la extracción asistida por microondas es una herramienta poderosa para la extracción de hormonas esteroideas en muestras sólidas, por lo que también se ha aplicado en este caso en muestras de tejido de peces. Los individuos estudiados fueron capturados en las inmediaciones del emisario submarino de una estación depuradora de aguas residuales que trata grandes volúmenes de agua residual urbana. Se eligió el estudio de organismos marinos cercanos al emisario ya que estos lugares concentran gran cantidad de vida debido a que son focos de materia orgánica. Al

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mismo tiempo, los emisarios submarinos son también focos de contaminación por compuestos emergentes como las hormonas esteroideas, por lo que el impacto que pueden crear en el ecosistema es importante.

En este trabajo se optimizaron las variables que afectan al método de extracción ya que, al tratarse de una matriz diferente, no son totalmente aplicables las condiciones previamente optimizadas para los lodos. Además, se ha tenido que añadir un paso de lavado de los extractos para eliminar los fosfolípidos y las proteínas extraídas, ya que constituyen una importante interferencia en el proceso de ionización en el equipo de espectrometría de masas. El método desarrollado, por tanto, se basa en extracción asistida por microondas, un paso de lavado realizado con extracción en fase sólida y la posterior separación y cuantificación de los analitos mediante cromatografía líquida de ultra resolución con detección por espectrometría de masas en tándem (MAE-SPE-UHPLC-MS/MS)

El método optimizado presenta una magnífica linealidad (coeficientes  $r^2$  superiores a 0,990), unos límites de detección muy adecuados para la matriz estudiada ( $0,14 - 49,0 \text{ ng}\cdot\text{g}^{-1}$ ) y buenas recuperaciones, del 56% de media para las muestras de músculo de pescado, y ligeramente inferiores para las muestras de vísceras y piel. La repetitividad del método fue bastante satisfactoria ya que se obtuvieron desviaciones estándar relativas *intra-day* e *inter-day* menores al 20%.

Una vez desarrollado, el método fue aplicado a muestras de diferentes tejidos de peces, para evaluar la presencia de hormonas en éstos. Por un lado, se evaluó la presencia de hormonas en muestras de músculo y de piel, por ser estas partes susceptibles de ser consumidas,

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mientras que, por otra parte, se aplicó el método a muestras de vísceras de los peces analizados debido a que estos tejidos son, a priori, los que pueden retener mayor cantidad de contaminantes. De esta forma se analizaron músculo, piel y vísceras de dos pequeños peces (*Boops boops* y *Sphoeroides marmoratus*) muy comunes en el litoral de las Islas Canarias y que constituyen la base de la cadena trófica.

En los diferentes tejidos analizados, se hallaron concentraciones de hasta ocho hormonas diferentes en concentraciones que llegaron hasta los  $3,95 \mu\text{g}\cdot\text{g}^{-1}$ . Concretamente, en las muestras de músculo se detectaron hasta siete hormonas diferentes, aunque todas ellas en concentraciones inferiores al límite de cuantificación, a excepción de la progesterona en *Sphoeroides marmoratus*, la cual se detectó a una concentración de  $0,56 \mu\text{g}\cdot\text{g}^{-1}$ . Por su parte, en las muestras de piel y vísceras de ambas especies se detectaron menos hormonas (entre tres y cuatro), pero a concentraciones superiores a las detectadas en las muestras de músculo.

El presente trabajo ha sido enviado a la revista *Food Chemistry* y se encuentra actualmente en fase de revisión.

## Capítulo 3. Parte experimental y resultados

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Manuscript Draft

Manuscript Number:

Title: DETERMINATION OF SEX HORMONES AND GLUCOCORTICOIDS IN FISH TISSUES BY MICROWAVE ASSISTED EXTRACTION AND SOLID PHASE EXTRACTION COUPLED TO ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Article Type: Research Article (max 7,500 words)

Keywords: Fish tissue; Mass spectrometry; Microwave-assisted extraction; Solid phase extraction; Steroid hormones; Ultra-high-performance liquid chromatography.

Corresponding Author: Professor José Juan Santana-Rodríguez, PhD

Corresponding Author's Institution: Universidad de Las Palmas de Gran Canaria

First Author: Rayco Guedes-Alonso, PhD Student

Order of Authors: Rayco Guedes-Alonso, PhD Student; Zoraida Sosa-Ferrera, PhD; José Juan Santana-Rodríguez, PhD

Abstract: Steroid hormones could produce adverse effects on biota as well as bioaccumulation in fish and seafood, so it is necessary the development of methodologies to determine these compounds in samples related to the food chain.

This work presents an analytical method for the determination of 15 steroid hormones in fish tissue. It is based on microwave-assisted extraction and solid phase extraction coupled to ultra-high-performance liquid chromatography tandem mass spectrometry (MAE-SPE-UHPLC-MS/MS). The proposed method shows appropriate detection limits (0.14 - 49.0 ng\*g<sup>-1</sup>), recoveries in the range of 50% and good reproducibility.

After optimization, the method was applied to different tissues of two small fishes of Canary Islands which constitute an important level of the food web (*Boops boops* and *Sphoeroides marmoratus*) and were exposed to the outfall of the Las Palmas de Gran Canaria wastewater treatment plant. The concentrations of eight detected compounds ranged from below quantification limits to 3.95 µg\*g<sup>-1</sup>.

### Cover Letter



Editor of Food Chemistry

Las Palmas de G.C. 12 February 2017

Dear Editor,

I'm enclosing you the manuscript entitled: DETERMINATION OF SEX HORMONES AND GLUCOCORTICOIDS IN FISH TISSUES BY MICROWAVE ASSISTED EXTRACTION AND SOLID PHASE EXTRACTION COUPLED TO ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY, for your consideration for publication in your Journal.

In this work we develop a microwave-assisted extraction (MAE) procedure for the extraction of fifteen steroid hormones in fish tissue samples from two kinds of fish from the coast areas of Canary Islands. According to the best of our knowledge, this is the first developed method that permits the determination of all types of steroid hormones in fish tissue based in microwave-assisted extraction. Additionally, solid phase extraction has been used as wash-step. The determination of these compounds has been carried out using ultra-high- performance liquid chromatography with tandem mass spectrometry detection (UHPLC-MS/MS). The developed method permits the analysis of biological samples in short times, with very good repeatability and producing small quantities of toxic residues, so it could be considered as a green chemistry methodology. Moreover, the small quantities of samples used allow the use of this method for the analysis of individual fishes or small communities. This could be very useful to evaluate isolated points of pollution or to compare the levels of exogenous steroid hormones in different species of fish.

All the parameters involved in the proposed methodology have been optimized and the method shows good linearity, repeatability and a really good selectivity, as well as low detection limits, appropriate for this type of compounds in biological samples. The optimized method has been successfully applied to the determination of these hormonal compounds in real samples. Taking into account its applicability, this methodology can be used as routine method for the control of these micropollutants in seafood.

In addition, we would like to indicate that English language of the manuscript has been corrected by a Language Editing Service of Elsevier. Please find enclosed the Certificate of language editing.

Sincerely yours,

Prof.Dr. José Juan Santana Rodríguez.  
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**To whom it may concern**

The paper "DETERMINATION OF SEX HORMONES AND GLUCOCORTICOIDS IN FISH TISSUES BY MICROWAVE ASSISTED EXTRACTION AND SOLID PHASE EXTRACTION COUPLED TO ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY" by JOSE JUAN SANTANA RODRIGUEZ was edited by Elsevier Language Editing Services.

Kind regards,

Biji Mathilakath  
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### **Highlights**

- Microwave-assisted extraction combined with UHPLC-MS/MS is proposed.
- The method is optimized and validated to determine fifteen steroid hormones.
- Extraction and determination variables have been optimized for target compounds from fish tissues
- Minimum quantities of sample and organic solvents and short extraction times are used.
- Fish tissue samples have been successfully analyzed using the proposed method.



## Capítulo 3. Parte experimental y resultados

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### DETERMINATION OF SEX HORMONES AND GLUCOCORTICOIDS IN FISH TISSUES BY MICROWAVE ASSISTED EXTRACTION AND SOLID PHASE EXTRACTION COUPLED TO ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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### 2 **Abstract**

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16

### 17 **Keywords**

18 Fish tissue, Mass spectrometry, Microwave-assisted extraction, Solid phase extraction,  
19 Steroid hormones, Ultra-high-performance liquid chromatography.

20

### 21 **1. Introduction**

22

23 For humans, seas and oceans are essential, not only to maintain the equilibrated climate over the  
24 world but also for many human activities and development. In fact, from 2005 to 2015, the  
25 United Nations developed the “International Decade for Action: Water for life” to achieve an  
26 international commitment about water issues.

27 Currently, water bodies and specifically oceans have become the spillway of the modern society  
28 because of their great regeneration ability (Bertinelli, Strobl, & Zou, 2008). Nevertheless, this  
29 regeneration capacity does not mean that the tons of organic compounds that reach rivers and

### Capítulo 3. Parte experimental y resultados

30 oceans every day are removed. Actually, aquatic ecosystems are greatly affected by these  
31 compounds, which many times have unknown side effects over aquatic biota (Ginebreda et al.,  
32 2014; Malaj et al., 2014). In the 2030 Agenda for Sustainable Development of the UN, “life  
33 below water” is one of the key topics (United Nations General Assembly, 2015), and also the  
34 organizations depending on UN as the Food and Agriculture Organization of the United Nations  
35 (FAO) consider this topic as a key for a sustainable future, because oceans are a basic source of  
36 food. For this reason, one aspect of this important topic could be the study of the quality of  
37 seafood knowing how and how much compounds affect aquatic organisms.

38 From the wide variety of emerging pollutants, sex hormones as well as corticosteroids are a  
39 worrisome group of compounds for the international scientific society because of their potential  
40 side effects upon aquatic biota. Some of these compounds (specifically three oestrogens) are  
41 now included on “Watch Lists” of international organizations such as the European Union to  
42 provide information about the concentrations of these emerging pollutants and give support to  
43 future environmental legislation (European Commission, 2015). The main concern relies on the  
44 documented capability of these contaminants to act as endocrine disruptors being able to  
45 interfere with the reproductive system and the normal development of living organisms.

46 Moreover, these endocrine disrupting compounds (EDCs) shows important bioaccumulation  
47 effects (Al-Ansari et al., 2010; Geyer et al., 2000), so the control of their concentrations in  
48 aquatic organisms is found to be absolutely crucial to ensure the quality of seafood.

49 The main sources of these pollutants into the environment are known to be the wastewater  
50 treatment plants (WWTP) (Deblonde, Cossu-Leguille, & Hartemann, 2011; Pal, Gin, Lin, &  
51 Reinhard, 2010) both because the treatments used in wastewater plants are not specifically  
52 designed to remove these types of compounds (Matamoros, Rodríguez, & Albaigés, 2016;  
53 Melvin & Leusch, 2016) and because of deconjugation of some hormones into other hormones  
54 produced by bacteria present in WWTPs (Belhaj et al., 2015; Liu, Lu, Yin, Dang, & Rittmann,  
55 2015). Other minor sources of sex hormones and corticosteroids in the environment are the  
56 runoff from cattle farming and from manure used in agriculture, as well as leaks and illegal  
57 disposal of domestic and industrial wastewater (Li, 2014).

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58 These discharges into the aquatic environment are an issue of concern for the scientific society  
59 and, subsequently, more and more studies connect changes in aquatic biota with the presence of  
60 anthropogenic organic compounds in the environment (Beyer et al., 2014; Muñoz et al., 2015).  
61 In addition, outfalls of WWTPs are known to be important zones of biodiversity because they are  
62 a source of a continuous organic matter flow, and, subsequently, are key feeding points of some  
63 small species that are the base of the food chain. Since the first studies of changes in aquatic  
64 biota due to the discharges of wastewater treatment plant effluent in the 1990s (Purdom et al.,  
65 1994), the studies of effects of different organic pollutants on biota have increased exponentially  
66 (Connon, Geist, & Werner, 2012). Of all types of organic pollutants, hormones are an important  
67 group because they are considered endocrine disrupting compounds and even at low  
68 concentrations, could produce changes in aquatic biota (Aris, Shamsuddin, & Praveena, 2014;  
69 Sumpter, 2005). Sex steroids activate and organize brain functions, even during foetal  
70 development (Pradhan & Olsson, 2015), so their presence in aquatic media is intimately linked  
71 to sexual changes in fish, hermaphroditism or unisexual populations (Colman, Baldwin,  
72 Johnson, & Scholz, 2009, p. 200; Svensson, Fick, Brandt, & Brunström, 2014). However, not  
73 only do steroid hormones affect sexual systems but they can also affect other biological  
74 systems. For example, Pradhan et al. (Pradhan & Olsson, 2015) have demonstrated that  
75 exposure of zebrafish to steroids resulted in gene changes that not only affected gonads but also  
76 produced changes in gene expression that resulted in brain dimorphisms and, subsequently,  
77 changes in fish behaviour. These changes in fish behaviour are also related to presence of some  
78 glucocorticoids such as cortisol, as stated by DiBattista et al. (DiBattista, Anisman, Whitehead,  
79 & Gilmour, 2005).

80 The development of analytical techniques that allow the extraction and concentration of sex  
81 hormones and glucocorticoids from biological tissues at trace concentrations is necessary  
82 because the effects of steroids on fish and aquatic biota could be produced even at  $\text{ng}\cdot\text{L}^{-1}$   
83 concentrations (Green et al., 2015; Schwindt, Winkelman, Keteles, Murphy, & Vajda, 2014)  
84 and those concentrations could suffer biomagnification through the whole food chain.  
85 Microwave-assisted extraction (MAE) has been used in the recent decades for digestion of solid

86 samples and for the extraction of organic pollutants from solid complex samples such as soils,  
87 sludges or tissues (Guedes-Alonso, Montesdeoca-Esponda, Sosa-Ferrera, & Santana-Rodríguez,  
88 2014). MAE could also be considered a green technique because of its low consumption of  
89 organic solvents and the capability to replace the organic solvents with biodegradable  
90 surfactants, a process known as microwave-assisted micellar extraction (MAME)  
91 (Montesdeoca-Esponda, Sosa-Ferrera, & Santana-Rodríguez, 2012). MAE has previously been  
92 used for the extraction of steroids from complex solid samples such as sludges, soils, biological  
93 tissues or foods (Dévier, Labadie, Togola, & Budzinski, 2010; Snow, Damon-Powell, Onanong,  
94 & Cassada, 2013; Yang, Li, Zhang, Sun, & Sun, 2014). However, the use of MAE for the  
95 extraction and pre-concentration of compounds from all families of steroids (oestrogens,  
96 androgens, progestogens and glucocorticoids) is not well developed, and only few articles have  
97 developed such a method (Guedes-Alonso et al., 2016; Wang et al., 2012).

98 Considering the information above, the aim of this work is to develop an analytical method for  
99 the extraction and determination of a group of 15 sex hormones and glucocorticoids of marine  
100 organisms. This methodology is based on microwave-assisted extraction and solid phase  
101 extraction as the purification step and ultra-high-performance liquid chromatography tandem  
102 mass spectrometry (MAE-SPE-UHPLC-MS/MS) as the determination system. A statistical  
103 experimental design has been used to develop the key variables that affect the extraction method  
104 and after optimization. This method has been used for the analysis of different fish tissue  
105 samples from two small and common fishes from the coasts of the Canary Islands.

## 106 **2. Materials and Methods**

### 107 2.1 Material, solvents and reagents

108 HPLC-grade methanol and acetonitrile, LC/MS-grade methanol, and LC/MS-grade water, as  
109 well as ammonia to adjust the pH of the mobile phase, were obtained from Panreac Química  
110 (Barcelona, Spain), while ultrapure water used was provided by a Milli-Q system (Millipore,  
111 Bedford, MA, USA). Diethylstilbestrol (DES), estrone (E1), 17 $\beta$ -estradiol (E2), 17 $\alpha$ -  
112 ethynylestradiol (EE), estriol (EE), levonorgestrel (NOR), norethisterone (NORET), megestrol  
113

114 acetate (MGA), progesterone (PRO), testosterone (TES), boldenone (BOL), nandrolone (NAN),  
115 cortisone (COR), prednisone (PRD) and prednisolone (PRDNL) were purchased from Sigma–  
116 Aldrich (Madrid, Spain) and showed purity over 99%. Stock solutions ( $1000 \text{ mg}\cdot\text{L}^{-1}$ ) of target  
117 analytes were prepared by dissolving the compound in methanol, and the solutions were stored  
118 in glass-stoppered bottles at  $-20^{\circ}\text{C}$  prior to use. Working standard solutions were prepared daily.

### 119 2.2 Sampling

120

121 To carry out the optimization of the conditions of the MAE, samples of the two fishes under  
122 study (*Sphoeroides marmoratus* and *Boops boops*) were fished in a pristine area of the sea east  
123 of Gran Canaria (Spain) to have individuals who had not been affected by wastewater effluents.  
124 The samples for the study once the method was optimized were fished in the proximity of the  
125 outfall of the wastewater treatment plant (WWTP) of the city of Las Palmas de Gran Canaria  
126 (Spain), which treats the wastewaters from a big urban area with a population equivalent to  
127 260,000. The coordinates of the exit of the outfall are 28.09292N, 15.39351 W. Skin, muscle,  
128 viscera and gills were separated from each individual and then the separate parts of each species  
129 were freeze-dried at a temperature of  $-55^{\circ}\text{C}$  and ground to obtain a powdered sample.

130

### 131 2.3 Instrumentation

132

133 For the extraction of the analytes, a Multiwave Microwave Sample Preparation System  
134 equipped with a 6 EVAP rotor and 6 MF100 vessels (Anton Paar, Graz, Austria) was used. For  
135 the purification step, the SPE cartridges tested were OASIS PRiME (Waters, Barcelona, Spain)  
136 and Phree Phospholipid removal (Phenomenex España, Madrid, Spain). The SPE wash steps  
137 were carried out in a Vac Elut 20 manifold from Varian (Agilent Technologies, California,  
138 USA) connected to a vacuum pump (Gast Manufacturing, Inc., Benton Harbor, MI, USA). The  
139 SPE was performed at a vacuum pressure of 20 bar. An ultra-high-performance liquid  
140 chromatography system coupled to a triple quadrupole mass spectrometer (UHPLC-MS/MS)  
141 was used as detection technique, and it consists of a quaternary pump to pulse the mobile phase,  
142 an autosampler capable of injecting up to 21 samples, a column oven and a triple quadrupole

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143 detector (TQD) with an electrospray interface (ESI), all from Waters (Barcelona, Spain). All the  
144 components were controlled with MassLynx Mass Spectrometry Software. The  
145 chromatographic separation was performed using an ACQUITY UHPLC BEH Waters C<sub>18</sub>  
146 analytical column (50 mm × 2.1 mm, 1.7 μm particle size) from Waters (Barcelona, Spain). All  
147 separation and detection parameters were developed in a previous article (Guedes-Alonso et al.,  
148 2016). Table S1 shows all parameters for MS/MS detection.

149  
150

### 151 2.4 Microwave-assisted extraction procedure

152

153 To perform the extraction of the target analytes, 100 mg of lyophilized fish tissue was placed in  
154 microwave polytetrafluoroethylene (PTFE) vessels, and 4 mL of methanol was added as the  
155 extractant. Then, the vessels were properly closed and placed in the microwave rotor in a  
156 symmetrical arrangement. Once the rotor was placed in the microwave, the extraction was  
157 conducted with the following program: in the first minute, the rotor spun without microwave  
158 power to prepare the samples for the extraction. After this minute, a power of 450 W for 7  
159 minutes was used to perform the extraction of the analytes from the tissue. Under these  
160 conditions, the methanol used as an extractant reached 60°C. Finally, the vessels were  
161 completely cooled for 5 minutes using the microwave fan and were then left at room  
162 temperature for another 10 minutes. Next, the extracts were filtered using 0.20 μm syringe  
163 polyethylene terephthalate (PET) filters from Macherey-Nagel (Düren, Germany). To clean the  
164 extracts, they were passed through Phenomenex Phree SPE cartridges to eliminate impurities  
165 such as phospholipids and proteins that affect the ionization in the UHPLC-MS/MS system.

## 166 3. Results and Discussion

167

### 168 3.1 Optimization of the microwave-assisted extraction

169

170 Some parameters such as sample weight, extractant volume, microwave power or extraction  
171 time could affect MAE. To evaluate how these parameters affect the extraction, an experimental

172 design was used. First, a  $2^4$  factorial experimental design means that the four variables under  
173 study at 2 different levels were used to study the interactions between variables as well as the  
174 significance of each variable in the extraction process. Then, the two variables that showed the  
175 greatest interaction were used in a  $3^2$  experimental design (2 variables at 3 levels) to determine  
176 the optimum extraction conditions. To compare the results of the different runs, triplicate spiked  
177 fish muscles (at an initial concentration of  $10 \mu\text{g}\cdot\text{g}^{-1}$ ) were used. The statistical analysis was  
178 performed using Minitab 17 Statistical Software.

179

### 180 3.1.1 $2^4$ factorial design

181

182 This factorial design allowed the contribution of the variables to be known, individually as well  
183 as together, during the extraction procedure. To avoid carryover effects, runs were randomized,  
184 and sample weight (100 and 500 mg), power (100 and 500 W), extraction time (2 and 10  
185 minutes) and extractant volume (4 and 10 mL of methanol) were studied. In Figure 1, Pareto  
186 charts show that power and extraction time are bound to the extraction of steroids from fish  
187 muscle. Not only are these variables significant individually for the extraction procedure but  
188 also the combination of them is significant. Keeping this in mind, these two variables were  
189 selected for the second part of the factorial design. Regarding the extractant volume of organic  
190 solvent, Pareto charts can be checked to show that this variable is not relevant for the extraction  
191 procedure. In addition, the media bivariate correlation for the target compounds was 0.01, which  
192 reasserted the results of the Pareto charts. For this reason, to maximize the pre-concentration  
193 power of the extraction technique, the extractant volume was set at 4 mL. Finally, the sample  
194 weight did not show an effect over the extraction procedure as strong as the power and  
195 extraction time. Moreover, the bivariate correlation for most compounds was negative, which  
196 indicates that larger sample weights produce a loss in the overall recovery in the extraction  
197 procedure, possibly because of the interferences present in a large sample amount rather than a  
198 small amount. For these reasons, 100 mg of fish tissue was fixed as the optimum value.

199

### 200 3.1.2 $3^2$ factorial design

201

202 The objective of the second factorial design was to refine the optimum value of the variables  
203 that are strongly bound to the extraction procedure. For this purpose, the two variables selected  
204 as the most dependent were studied at three levels, doing a factorial design of 11 randomized  
205 runs (9 interactions with a triplicate of the central point). The studied levels were 100, 500 and  
206 700 W of microwave power and extraction times of 2, 6 and 10 minutes. Higher powers and  
207 times were not tested because over 700 W and 10 minutes, the solvent was almost evaporated  
208 producing non-reproducible replicates. As Figure 2 shows, response surfaces for different target  
209 compounds show some differences between the compounds but in most cases, present  
210 maximum or, at least, high values in the ranges of 350 – 500 W and 6 – 8 minutes. The values  
211 fixed as optimum for the extraction were 450 W of irradiation power during 7 minutes.

212

### 213 3.2 Evaluation of extraction solvent

214

215 In the extraction of organic compounds from animal or human tissue, the choice of an  
216 appropriate extraction solvent is a key factor because not only must the extraction solvent be  
217 suitable for the total extraction of the compounds from the tissue but also the extraction of  
218 interferences must be as little as possible. In this regard, methanol and acetonitrile and 50:50  
219 (v/v) mixtures of them with water were analysed as extractants, evaluating the recoveries  
220 obtained as well as the signal suppressions produced in comparison with a standard (Figure S1).  
221 Acetonitrile produced poor recoveries, in all cases below 15%. For the mixture ACN:water  
222 (50:50, v/v) the recoveries were higher, but the signal suppressions were significant too because  
223 they were over 30% in most cases. Regarding methanol, the differences using pure methanol or  
224 a mixture 50:50 (v/v) with water were not very significant and the recoveries were over 50% for  
225 most compounds. In fact, the mixture methanol:water extracted the corticosteroids, megestrol  
226 acetate and nandrolone better, while pure methanol was more effective with the oestrogens and  
227 the rest of the androgens and progestogens. Moreover, the signal suppressions produced by the  
228 mixture of methanol and water as extractant were twice as higher in most cases as the

229 suppressions produced by pure methanol. In fact, the median ion suppression of the mixture was  
230 36% while the median ion suppression for pure methanol was 16%. Therefore, pure methanol  
231 was selected as extractant solvent for MAE.

232

### 233 3.3 Wash step and matrix effect evaluation

234

235 Extraction of organic compounds from complex matrices such as organic tissues is known to be  
236 very challenging when LC-MS/MS is used as determination technique because of the great  
237 quantity of interferences that produce a loss in the analytical signal. Phospholipids and proteins  
238 are two types of compound that could affect the good ionization of compounds in MS/MS and,  
239 subsequently, produce a loss in the analytical signal (Guo & Lankmayr, 2011; Van Eeckhaut,  
240 Lanckmans, Sarre, Smolders, & Michotte, 2009). Moreover, when methanol is used as the  
241 organic solvent extractant instead of acetonitrile, the signal losses produced by phospholipids  
242 could be higher (Lahaie, Mess, Furtado, & Garofolo, 2010). Therefore, to avoid this problem,  
243 some commercial SPE cartridges are specifically designed for the removal of phospholipids and  
244 proteins from extracts of biological samples. Most of the cartridges are also designed to remove  
245 these types of compounds from organic solvent extracts such as the cartridges tested in this  
246 work. Figure 3 shows the changes in the analytical signal produced by the two SPE cartridges  
247 selected using methanol and a mixture 50:50 (v/v) of methanol:water. These changes were  
248 obtained comparing the signals of MAE and MAE-SPE extracts. The changes (losses or  
249 enhancements) were more noticeable when water was present as the extractant solvent while the  
250 extractions using pure methanol presented changes in the range of  $\pm 10\%$ . Regarding the signal  
251 suppression or enhancement of the signal after the wash step, Figure 3 shows that Phenomenex  
252 Phree cartridges produced lower signal suppressions than OASIS PRiME cartridges. For all of  
253 the above reasons, Phenomenex Phree cartridges were selected for the wash step and methanol  
254 as extractant solvent was reasserted as optimum. The algorithm used by Vieno et al. (Vieno,  
255 Tuhkanen, & Kronberg, 2006) was used to evaluate the changes in the analytical signal caused  
256 by matrix effects.

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257 To resolve the matrix effects of the samples, three internal standards were added to the MAE  
258 extracts after the wash step at a concentration of  $200 \mu\text{g}\cdot\text{L}^{-1}$ . Deuterated compounds were  
259 chosen as internal standards: oestrone-D2 for the analysis of oestrogens, testosterone-D3 for  
260 androgens and progesterone-D9 for the analysis of progestogens and glucocorticoids.

261

### 262 3.4 Analytical parameters and quality control

263

264 Analytical parameters for the proposed method have been developed. Linearity, detection and  
265 quantification limits, recoveries and reproducibility were evaluated for the different matrices  
266 studied in this work. The method demonstrates a very good linearity for the compounds under  
267 study in the external calibration curves used. These external curves were built using the relation  
268 between areas and concentrations of the compounds studied and their respective internal  
269 standards that were at a fixed concentration of  $200 \mu\text{g}\cdot\text{L}^{-1}$ . For all the compounds, correlation  
270 coefficients ( $r^2$ ) were higher than 0.990.

271 Detection and quantification limits (LOD and LOQ, respectively) were calculated as the  
272 concentration that generated a signal-to-noise ratio of 3 and 10 in the quantification ion  
273 transition. LODs and LOQs are observed to be higher in viscera and skin because there were  
274 more interferences in these matrices, producing a loss in the overall recovery, and subsequently,  
275 higher analytical limits. Nevertheless, the limits reached are adequate for the analysis of these  
276 types of compounds in biological samples and similar to the limits reached with other extraction  
277 and detection techniques (Ros, Vallejo, Olivares, Etxebarria, & Prieto, 2016; Watson, Potter,  
278 Murphy, & Gibbs, 2015).

279 The recoveries were studied by comparing the signals of extracts of spiked sludge samples with  
280 extracts of non-spiked samples, spiked after the MAE-SPE method. The recoveries were studied  
281 at two concentration levels ( $0.4$  and  $4 \mu\text{g}\cdot\text{g}^{-1}$ ). Figure 4 shows the results obtained. The figure  
282 shows that the recoveries from fish muscle tissue were higher than the recoveries in the other  
283 two matrices studied. The median recoveries from muscle were approximately 56%, while for  
284 viscera and skin were 30% lower.

285 Intra- and inter-day repeatability of the method was evaluated using three samples per day  
286 (Table 1). The samples were spiked with target compounds at the same three concentration  
287 levels. Both repeatability values were satisfactory, and the relative standard deviations were, in  
288 all cases, below 20%.

289

290 3.5 Analysis of hormonal compounds in tissue samples

291

292 The proposed method was applied to tissues from two very common fishes from the Canary  
293 island coast. The chosen species (*Boops boops* and *Sphoeroides marmoratus*) are  
294 acanthopterygii bony fishes, of a maximum length of 20 centimetres and very common near the  
295 coast (up to 100 metres of depth). Both species are omnivorous but the main difference is that  
296 *Sphoeroides marmoratus* finds its food in the sand, while *Boops boops* feeds on both water  
297 bodies and sediments. Both species have been chosen because they are usually near  
298 anthropogenic sources of organic matter such as the outfalls of wastewater treatment plants and,  
299 subsequently, can be considered as biomarkers for the water quality.

300 Table 2 shows the concentrations detected in the different parts of individuals of both species.

301 Muscle tissue of both species showed more detected compounds but at very low concentrations  
302 that were under the detection limits while viscera and skin samples showed fewer detected  
303 compounds but at higher concentrations. Only progesterone was detected in all tissues analysed  
304 at concentrations up to  $3.95 \mu\text{g}\cdot\text{g}^{-1}$ . Moreover, norgestrel and nandrolone were detected and  
305 quantified in viscera tissue from both species under study in concentrations ranging from 0.07 to  
306  $2.71 \mu\text{g}\cdot\text{g}^{-1}$ . One corticosteroid, prednisolone, was detected in *Boops boops* skin at a  
307 concentration of  $0.09 \mu\text{g}\cdot\text{g}^{-1}$ . It is important to state that no oestrogens were detected in any  
308 sample under study.

309

310 **4. Conclusions**

311

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312 The method developed is based on microwave assisted extraction and solid phase extraction as  
313 the wash step, and ultra-high-performance liquid chromatography tandem mass spectrometry as  
314 the determination technique (MAE-SPE-UHPLC-MS/MS) enables easy and quick  
315 determination of both sex hormones and glucocorticoids from fish tissue in less than 30  
316 minutes. The method shows appropriate detection and quantification limits as well as good  
317 linearity and repeatability. After the optimization of the variables of the extraction method, the  
318 method was applied to real samples from two common fishes from the coasts of the Canary  
319 Islands, *Boops boops* and *Sphoeroides marmoratus* which are two of the species that are in the  
320 base of the food chain. Despite the differences between the two species under study, the  
321 concentrations detected in the three different tissues analysed were similar and comparable. For  
322 both species, the higher concentrations of detected compounds were in viscera and skin while  
323 the concentrations in muscle tissue were lower. The concentrations of detected compounds  
324 ranged from below quantification limits to  $3.95 \mu\text{g}\cdot\text{g}^{-1}$ .

325

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330

### 331 **Conflict of interest**

332 The authors declare that they have no competing interests.

333

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335

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493

494

495 **Figure caption:**

496 **Figure 1.** Pareto charts of standardized effects of the variables studied in the 2<sup>4</sup> experimental  
497 design of a compound from each steroid hormones family under study.

498

499 **Figure 2.** Surface responses of the variables studied in the 3<sup>2</sup> experimental design of a  
500 compound from each steroid hormones family under study.

501

502 **Figure 3.** Evaluation of wash-step by: a) changes in the analytical signal and b) signal  
503 enhancement/suppression.

504

505 **Figure 4.** Calculated recoveries for the different tissues under study at two concentration levels.

506

507 **Figure S1.** Evaluation of extractant solvents for MAE by: a) recoveries and b) analytical signal  
508 changes.

509

510

Table 1

**Table 1.** Analytical parameters for the different tissues analysed with the proposed method

Compound	Linearity ( $r^2$ )	Muscle			Skin			Viscera		
		LOD <sup>a</sup> (ng·g <sup>-1</sup> )	Intra-day RSD <sup>b</sup> (%) 4 µg·g <sup>-1</sup>	LOD <sup>a</sup> (ng·g <sup>-1</sup> )	Intra-day RSD <sup>b</sup> (%) 4 µg·g <sup>-1</sup>	LOD <sup>a</sup> (ng·g <sup>-1</sup> )	Intra-day RSD <sup>b</sup> (%) 4 µg·g <sup>-1</sup>	LOD <sup>a</sup> (ng·g <sup>-1</sup> )	Intra-day RSD <sup>b</sup> (%) 4 µg·g <sup>-1</sup>	LOD <sup>a</sup> (ng·g <sup>-1</sup> )
DES	0.9977	1.60	0.71	4.69	15.3	9.82	1.84	2.43	15.8	14.4
EE	0.9963	11.8	28.9	2.35	11.8	5.61	14.9	3.72	20.0	2.91
E2	0.9900	29.8	94.1	1.22	19.5	11.5	2.82	3.12	3.86	15.2
E1	0.9973	2.99	17.4	11.3	7.23	11.5	16.6	2.03	8.95	9.68
E3	0.9992	5.35	19.7	14.8	11.6	16.2	3.14	5.17	17.6	1.43
NOR	0.9968	1.78	1.70	3.22	16.0	7.52	12.5	17.2	7.67	6.47
TES	0.9981	1.19	2.47	3.17	2.64	4.28	10.4	3.27	7.45	5.75
MGA	0.9976	2.21	7.91	6.22	0.14	11.2	10.8	1.05	23.0	2.69
PRD	0.9965	3.43	19.5	5.67	49.0	7.41	15.6	1.36	23.8	7.06
PRDNL	0.9952	0.72	1.01	5.59	42.9	18.1	2.74	18.8	15.9	3.95
COR	0.9961	2.88	6.99	6.15	8.29	2.64	13.1	2.50	1.43	0.85
BOL	0.9972	1.46	9.96	7.65	2.73	4.89	9.71	1.77	6.89	1.41
NORET	0.9983	5.04	14.7	3.76	11.8	6.06	11.8	15.3	11.3	5.51
NAN	0.9964	3.78	9.56	8.26	7.71	12.5	10.5	18.5	9.81	1.28
PRO	0.9966	2.40	13.7	6.37	2.29	6.92	8.91	3.06	1.90	3.86

<sup>a</sup> Limit of detection

<sup>b</sup> Relative standard deviation (n=3)

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Table 2

**Table 2.** Concentrations ( $\mu\text{g}\cdot\text{g}^{-1}$ ) of detected compounds in the different samples studied (n=3) from two fish species.

Compound	<i>Boops boops</i>			<i>Sphoeroides marmoratus</i>		
	Muscle	Viscera	Skin	Muscle	Viscera	Skin
NOR	< LOQ <sup>a</sup>	2.71	n.d.	< LOQ	0.50	n.d.
TES	< LOQ	n.d.	n.d.	< LOQ	n.d.	n.d.
MGA	< LOQ	n.d.	< LOQ	< LOQ	n.d.	< LOQ
PRDNL	n.d. <sup>b</sup>	n.d.	0.09	n.d.	n.d.	n.d.
BOL	< LOQ	< LOQ	n.d.	< LOQ	< LOQ	n.d.
NORET	< LOQ	n.d.	n.d.	< LOQ	n.d.	n.d.
NAN	< LOQ	0.52	n.d.	n.d.	0.07	n.d.
PRO	< LOQ	3.22	1.77	0.56	3.26	3.95

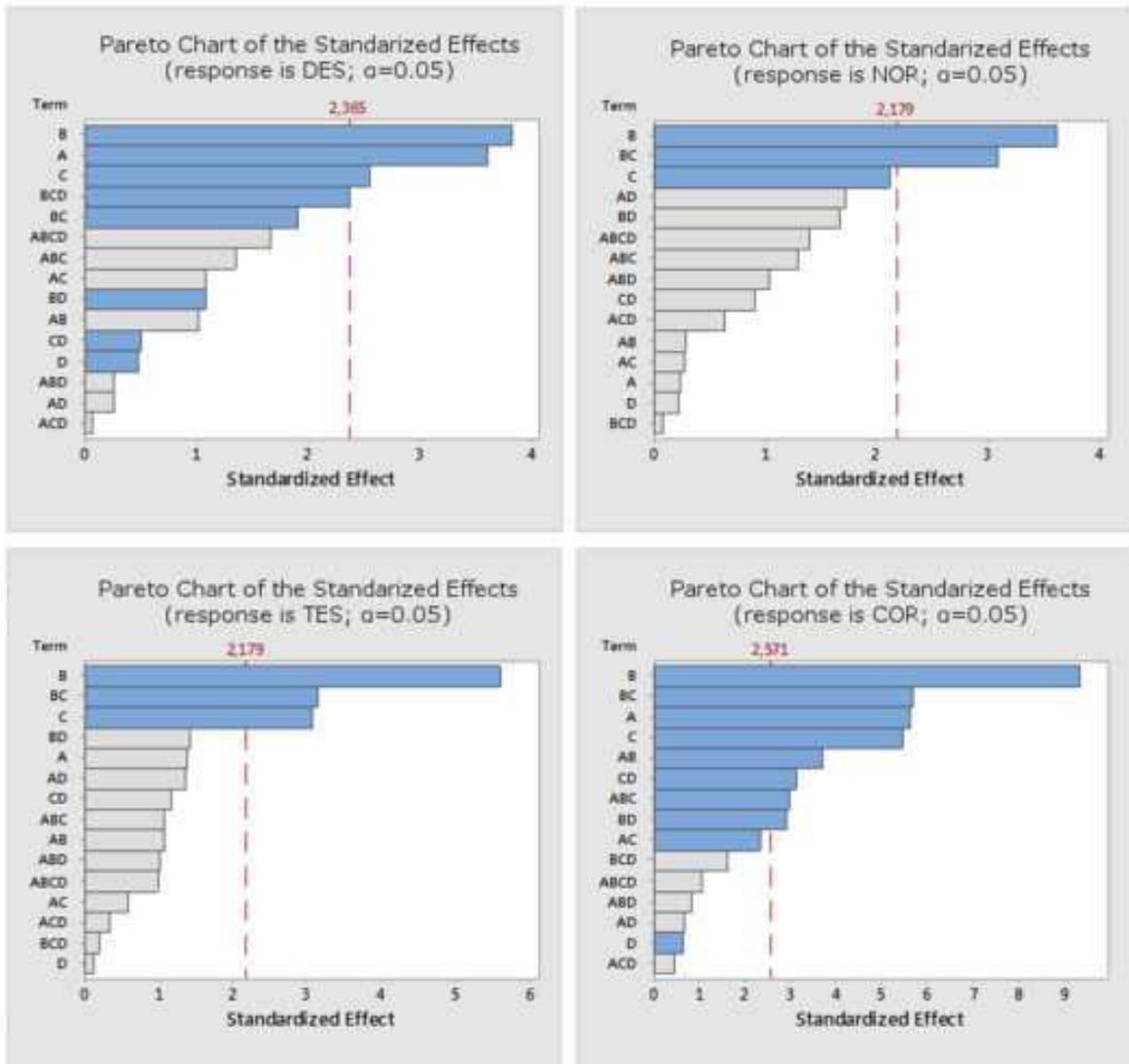
<sup>a</sup>Concentration under quantification limit.

<sup>b</sup>Not detected.

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Figure 1

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A: Sample weight (mg)  
B: Power (W)  
C: Extraction time (min)  
D: Extractant volume (mL)

## Capítulo 3. Parte experimental y resultados

Figure 2

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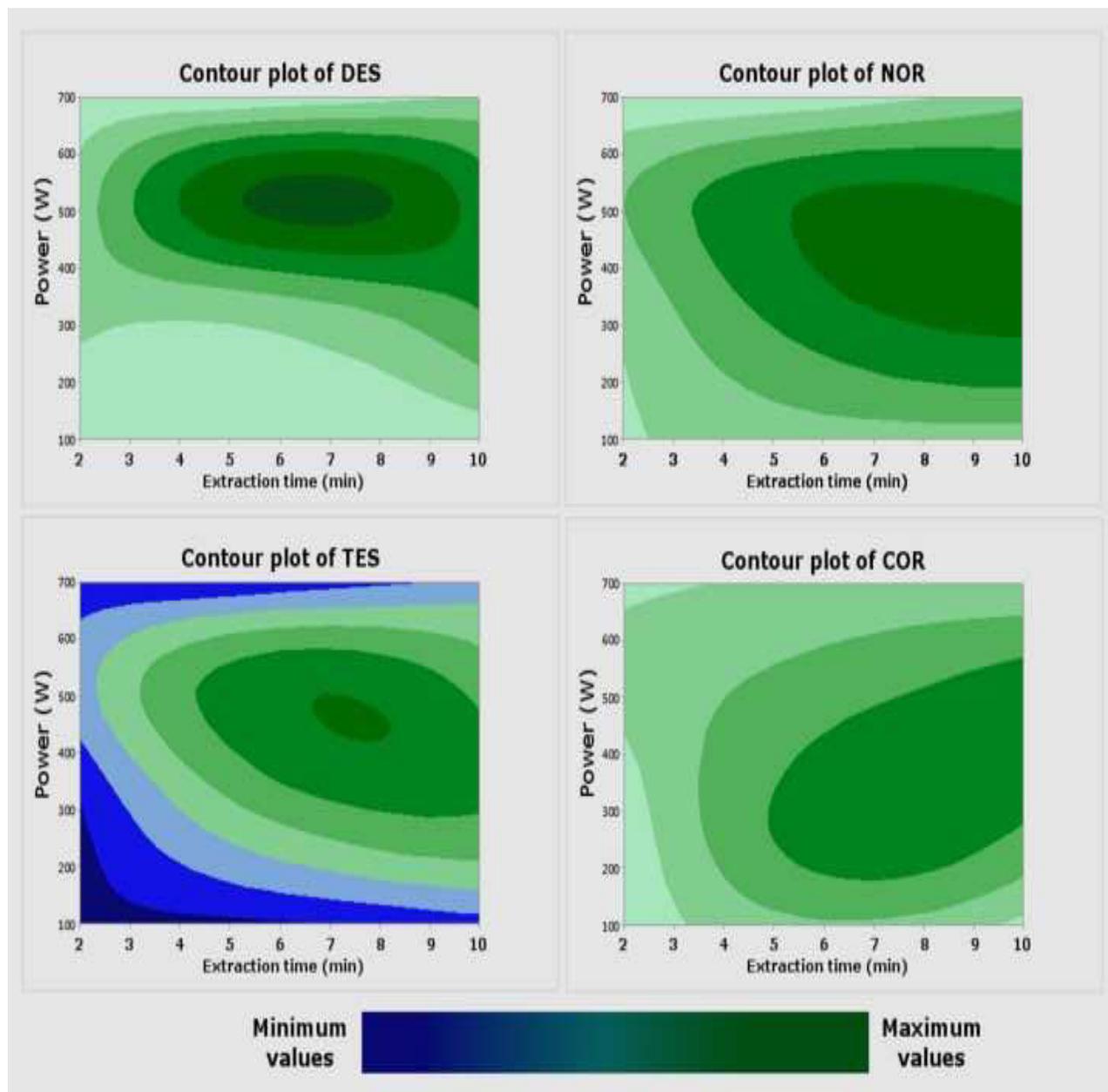
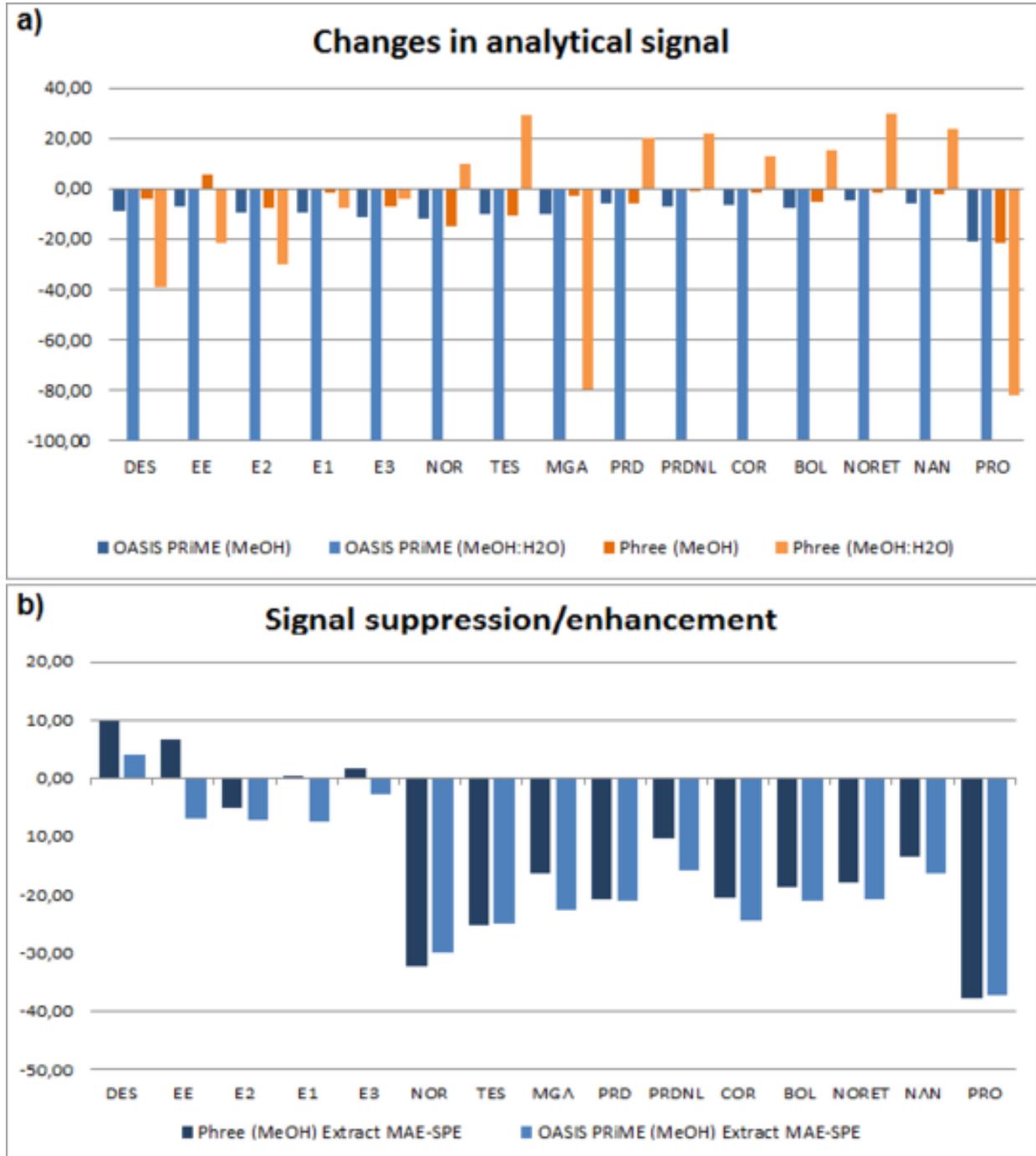
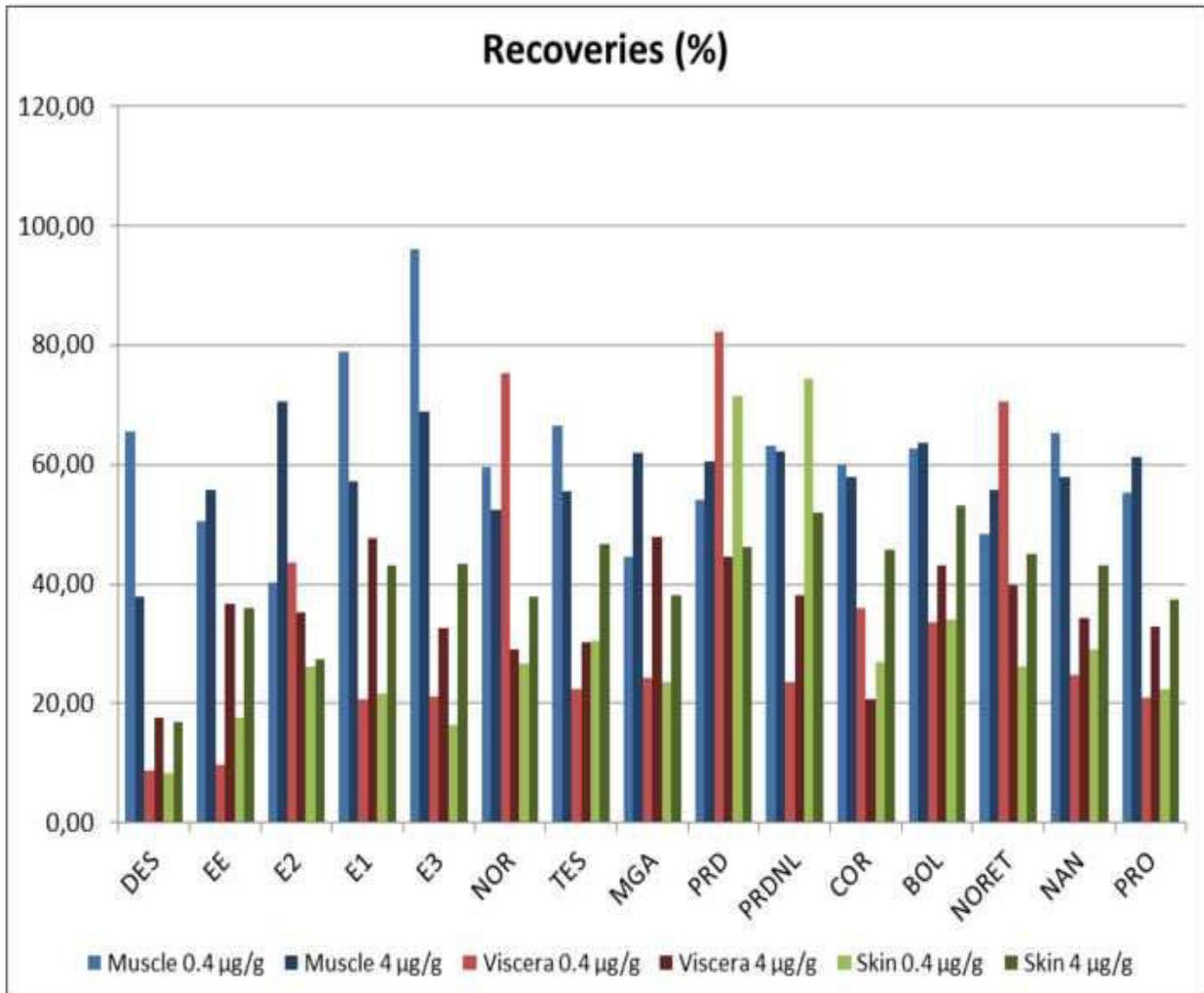


Figure 3  
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### Capítulo 3. Parte experimental y resultados

Figure 4  
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## Capítulo 4. Conclusiones

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Del conjunto de trabajos realizados y descritos en la presente Tesis Doctoral se pueden extraer las siguientes conclusiones:

1. De la optimización y desarrollo de las metodologías de extracción:
  - a. El método de extracción basado en SPE tanto para estrógenos fluorescentes como para un grupo de ocho hormonas esteroideas presenta parámetros analíticos adecuados para la extracción y preconcentración de hormonas en muestras reales.
  - b. La metodología de SPE en modalidad *on-line* optimizada permite la extracción, preconcentración y análisis de muestras reales en menos de 15 minutos con una mínima manipulación de las muestras. El método desarrollado presenta recuperaciones comparables a los métodos de SPE tradicionales, las cuales se encuentran entre el 50 y el 90% así

## Capítulo 4. Conclusiones

como extraordinarias repetibilidades (RSDs inferiores al 18%) y límites de detección (entre 0,5 y 13,2 ng·L<sup>-1</sup>)

- c. La comparación del método de extracción en fase sólida *on-line* con los métodos de SPE convencionales demostró que el acoplamiento de esta técnica de extracción a la cromatografía líquida de ultra resolución permite conseguir factores de preconcentración muy superiores a los obtenidos en la SPE tradicional y además, presenta la ventaja añadida de que el análisis puede ser llevado a cabo utilizando únicamente 2 mL de muestra de agua residual, un volumen 125 veces menor que el utilizado en la SPE convencional u *off-line*.
- d. La utilización de polímeros impresos molecularmente como adsorbentes en la extracción en fase sólida se mostró como una alternativa altamente selectiva para la extracción y preconcentración de estrógenos en muestras líquidas. La utilización de esta fase adsorbente proporcionó un método con recuperaciones (superiores al 60%) y repetibilidades (desviaciones estándar relativas inferiores al 10%) comparables a la de la SPE tradicional. Asimismo, se mostró como un método altamente selectivo pues se extraían muchas menos interferencias que con polímeros tradicionales.
- e. Una nueva metodología, conocida como microextracción por adsorción sobre dispositivos químicamente modificados (FPSE) se aplicó a la extracción de andrógenos y progestágenos presentes en diferentes tipos de muestras líquidas. Este procedimiento proporcionó recuperaciones

superiores al 70% y límites de detección comparables a los obtenidos con técnicas de extracción convencionales. Esta técnica miniaturizada permitió la extracción de diez hormonas diferentes utilizando volúmenes de muestra muy pequeños (10 mL en el caso de aguas residuales y 20 mL para muestras de orina) y su análisis en tiempos relativamente cortos, de menos de 30 minutos.

- f. La comparación de la extracción por adsorción sobre dispositivos químicamente modificados con técnicas de extracción tradicionales reveló que la FPSE presenta un enorme potencial y que puede ser una alternativa a aquellas, presentando recuperaciones relativas superiores al 70%.
- g. La técnica basada en la extracción asistida por microondas seguida de un paso de limpieza por extracción en fase sólida para la eliminación de interferencias como fosfolípidos y proteínas (MAE-SPE) ofreció resultados satisfactorios para las hormonas esteroideas estudiadas en términos de eficiencia de extracción, así como de limpieza de extracto. Esta metodología se aplicó a muestras sólidas complejas, obteniéndose buenas recuperaciones, superiores al 50%, altas reproducibilidades y la capacidad de extraer hormonas esteroideas pertenecientes a cuatro de las familias en las que aquellas se dividen.

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2. De la optimización y desarrollo de las metodologías de separación y determinación:
  - a. Las técnicas basadas en cromatografía líquida de ultra resolución (UHPLC) proporcionan una separación cromatográfica extraordinaria de los diferentes compuestos hormonales en estudio a pesar de sus estructuras moleculares y propiedades similares.
  - b. La utilización de detectores de fluorescencia en compuestos hormonales que presentan esta característica como los estrógenos, se reveló como una alternativa a sistemas de detección de mayor coste. De hecho, los métodos basados en SPE y cromatografía líquida de ultra resolución con detección por fluorescencia, presentaron límites de detección competitivos, de hasta  $0,1 \text{ ng}\cdot\text{mL}^{-1}$ , en muestras complejas como las aguas depuradas.
  - c. La utilización de la espectrometría de masas como técnica de detección en modalidad “*multiple reaction monitoring*” produjo métodos de análisis altamente sensibles, capaces de detectar concentraciones iguales a las décimas de partes por trillón ( $\text{ng}\cdot\text{L}^{-1}$ ). Al mismo tiempo, el uso de la espectrometría de masas de triple cuadrupolo (MS/MS) permitió además la extraordinaria cuantificación de hormonas esteroideas con masas moleculares muy similares, o incluso iguales.
  - d. La combinación de la cromatografía líquida de ultra resolución con la espectrometría de masas en tándem ha sido la técnica de determinación que ha proporcionado mejor separación cromatográfica, identificación de compuestos y

sensibilidad del método de análisis, por lo que se considera muy idónea para la determinación de hormonas esteroideas en muestras medioambientales.

3. De la aplicación de las metodologías desarrolladas a muestras reales:

a. Por primera vez se ha podido cuantificar la presencia de hormonas esteroideas en muestras procedentes de diferentes compartimentos ambientales de la isla de Gran Canaria. Los niveles de hormonas detectadas han sido los siguientes:

- Aguas residuales procedentes de estaciones depuradoras de aguas residuales de zonas urbanas densamente pobladas:  $3,1 - 52,8 \text{ ng}\cdot\text{L}^{-1}$  en los efluentes de las EDARs.
- Aguas residuales de un complejo hospitalario veterinario de la isla de Gran Canaria:  $1,63 \text{ }\mu\text{g}\cdot\text{L}^{-1}$  de estriol
- Aguas residuales procedentes de dos pequeñas estaciones depuradoras de aguas residuales de zonas rurales:  $35 - 300 \text{ ng}\cdot\text{L}^{-1}$  en los influentes y pequeñas concentraciones (inferiores a  $50 \text{ ng}\cdot\text{L}^{-1}$ ) de algún estrógeno en los efluentes.
- Aguas sin tratar de una zona hospitalaria de la ciudad de Las Palmas de Gran Canaria:  $227,3 \text{ ng}\cdot\text{L}^{-1}$  de progesterona y concentraciones por debajo del límite de cuantificación de acetato de megesterol, boldenona y testosterona.

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- Muestras de orina de hombres sanos:  $1.1 \mu\text{g}\cdot\text{L}^{-1}$  de progesterona y  $2,3 \mu\text{g}\cdot\text{L}^{-1}$  de testosterona
  - Lodos procedentes de diferentes estaciones depuradoras de aguas residuales: entre 17,3 y 1140  $\text{ng}\cdot\text{g}^{-1}$  de etinilestradiol, levonorgestrel o cortisona.
  - Tejidos de peces capturados en las inmediaciones del emisario submarino de la mayor EDAR de la isla de Gran Canaria: hasta ocho hormonas esteroideas diferentes en concentraciones que llegaron hasta los  $3,95 \mu\text{g}\cdot\text{g}^{-1}$
- b. La eliminación de hormonas esteroideas producida en dos pequeñas estaciones depuradoras de aguas residuales de zonas rurales fue prácticamente del 100%, tanto usando tratamientos tradicionales de lodos activos como utilizando nuevas metodologías de depuración como los biorreactores de membrana.
- c. Las características hidrófobas de los lodos generados en EDAR con tratamientos de biorreactor de membrana provocan que las concentraciones de hormonas detectadas en este tipo de lodos sean sustancialmente mayores que las encontradas en lodos de EDAR basadas en tratamientos de lodos activos.
- d. Las concentraciones de hormonas detectadas en diferentes tejidos de pequeños peces expuestos a emisarios submarinos de EDAR son mayores en las vísceras y piel de éstos que en el tejido muscular.

## Capítulo 4. Conclusions

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The following conclusions could be extracted from the works described in this Doctoral Thesis:

1. About the optimization and development of extraction methodologies:
  - a. Solid phase extraction method used to extract both fluorescent oestrogens and a group of eight different steroid hormones presents appropriate analytical parameters for the extraction and preconcentration of hormones from real samples.
  - b. The optimised on-line solid phase extraction methodology permits the extraction, preconcentration and analysis of real samples in less than 15 minutes with a minimum sample handling. The developed method has similar recoveries (between 50 and 90%) as traditional SPE, extraordinary

## Capítulo 4. Conclusiones

repeatability (RSDs below 18%) and appropriate detection limits (0.5 – 13.2 ng·L<sup>-1</sup>).

- c. The comparison between on-line and traditional SPE methods demonstrates that the coupling of this extraction technique to an ultra-high performance liquid chromatography system permits to achieve preconcentration factors higher than those obtained using traditional SPE. Moreover, on-line SPE could perform a whole analysis using only 2 mL of sample (125 times lower than traditional SPE)
- d. The use of molecularly imprinted polymers such as SPE sorbents resulted in a very selective option to carry out the extraction and preconcentration of oestrogens in liquid samples. Using this new sorbent phase, the method showed recoveries (higher to 60%) and a repeatability (RSDs lower of 10%) similar to traditional SPE. Moreover, the method showed a very good selectivity because if MIPs are used, the extracted interferences are much lower.
- e. A new microextraction methodology known as fabric phase sorptive extraction (FPSE) was optimized to extract androgens and progestogens from liquid samples. The recoveries of the extraction method were higher than 50% y the detection limits were similar to those obtained in traditional extraction techniques. The miniaturized technique was successfully applied to samples in order to extract 10 different hormones using only 10 mL of wastewater samples and 20 mL of urine samples. The extraction times were relatively short, using less than 30 minutes.

- f. The comparison of fabric phase sorptive extraction and traditional extraction techniques as SPE showed that FPSE is a robust technique and could be an alternative of traditional ones, because the relative recoveries were over 70%.
  - g. Microwave assisted extraction (MAE) followed by solid phase extraction as wash step for the removal of proteins and phospholipids was an appropriate method for the extraction of steroid hormones from complex solid matrices. In fact, the obtained recoveries were over 50% and the method was highly repeatable. Moreover, the method was capable to extract hormones from four subfamilies from solid samples.
2. About the optimization of separation and determination methodologies:
- a. Ultra-high performance liquid chromatography is a powerful analytical tool, which provides excellent chromatographic separation of steroid hormones, despite the similar molecular structures and physicochemical properties of them.
  - b. The use of fluorescence detectors in the analysis of fluorescence hormones (mainly oestrogens) is an alternative to more expensive detection systems. In fact, the methods based in SPE and UHPLC with fluorescence detection showed detection limits from up to  $0.1 \text{ ng}\cdot\text{mL}^{-1}$  in complex liquid matrices as wastewaters.
  - c. Using mass spectrometry as detection technique in *multiple reaction monitoring*, it could be possible to detect concentrations of tenth parts-per-trillion. Moreover, the use of triple quadrupole mass spectrometry detection allowed for

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the quantification of steroid hormones with very similar molecular weight, and even the same.

- d. The combination of ultra-high performance liquid chromatography and triple quadrupole mass spectrometry was a powerful technique which has provided the best chromatographic separation, compounds identification and sensitivity. Because of this it is considered as an ideal technique for the determination of steroid hormones in environmental samples.
3. About the application of the developed methodologies to real samples:
    - a. For the first time it can be quantified the presence of steroid hormones in samples from the different environmental compartments of Gran Canaria Island.
      - Wastewater from high-density urban areas: 3.1 – 52.8 ng·L<sup>-1</sup> in WWTP effluent samples.
      - Veterinary-hospital wastewaters from Gran Canaria: 1.63 µg·L<sup>-1</sup> of estriol.
      - Wastewater from two small wastewater treatment plants located in rural areas: 35 – 300 ng·L<sup>-1</sup> in influent samples and concentrations below 50 ng·L<sup>-1</sup> of some oestrogens in effluent samples.
      - Raw wastewater from a hospital area of the city of Las Palmas de Gran Canaria: 227.3 ng·L<sup>-1</sup> of progesterone and concentrations below quantification limits of megesterol acetate, boldenone and testosterone.

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- Urine samples of healthy men:  $1.1 \mu\text{g}\cdot\text{L}^{-1}$  of progesterone and  $2.3 \mu\text{g}\cdot\text{L}^{-1}$  of testosterone.
  - Sludge samples from different wastewater treatment plants: between 17.3 and  $1140 \text{ ng}\cdot\text{g}^{-1}$  of ethynylestradiol, levonorgestrel and cortisone.
  - Different tissues of fishes caught in the proximity of the marine outfall of the biggest wastewater treatment plant of Gran Canaria: up to eight steroid hormones at concentrations up to  $3.95 \mu\text{g}\cdot\text{g}^{-1}$
- b. The removal of steroid hormones in the small WWTPs located in rural zones were almost 100% using conventional treatments of activated sludge as well as new purification systems as membrane bioreactors.
- c. The hydrophobicity of membrane bioreactor sludge produces that the concentrations of steroid hormones in this type of sludge are higher than the concentrations in activated sludges.
- d. Steroid hormones concentrations detected in fish tissue samples reveal that the concentrations in viscera and skin are higher than those in muscle tissue.



## Anexo I. Acrónimos

**CDEs:** Compuestos disruptores endocrinos

**CE:** Comisión Europea

**DEs:** Disruptores endocrinos

**DHEA:** Dehidroepiandrosterona

**DHT:** 5 $\alpha$ -dihidrotestosterona

**DLLME:** Dispersive liquid-liquid microextraction

**EC<sub>50</sub>:** Dosis con efectos media

**EDAR:** Estación depuradora de aguas residuales

**EDCs:** Endocrine disruptor compounds

**EEF:** Estradiol equivalent factor

**EEQ:** Estradiol equivalent quotient

**EIC:** Expected Introductory Concentration

**ELISA:** Enzyme-Lynked ImmunoSorbent Assay

**EMA:** European Medicines Agency

## *Anexos*

**EPs:** Emerging pollutants

**ER:** Estrogenic receptor

**ESI:** Electrospray ionization

**FDA:** Food and Drug Administration

**FPSE:** Fabric phase sorptive extraction

**GC:** Gas chromatography

**HF-LPME:** Hollow fiber liquid-phase microextraction

**HPLC:** High-performance liquid chromatography

**LC:** Liquid chromatography

**LC<sub>50</sub>:** Dosis letal media

**LLE:** Liquid-liquid extraction

**LOD:** Limit of detection

**LOQ:** Limit of quantification

**MAE:** Microwave-assisted extraction

**MEEC:** Maximal estimated environmental concentration

**MIP:** Molecular imprinted polymer

**MISPE:** Molecularly imprinted solid phase extraction

**MS:** Mass spectrometry

**MS/MS:** Triple quadrupole mass spectrometry

**NAMS:** North American Menopause Society

**OMS:** Organización Mundial de la Salud

**PBTs:** Persistent, bioaccumulative and toxic substances

**PEC:** Predictive environmental concentration

**PLE:** Pressurized liquid extraction

**PNEC:** predicted no-effect concentration

**PPCPs:** Pharmaceuticals and personal care products

**PS-DVB:** Poliestireno – divinilbenceno

**QuEChERS:** Quick, easy, cheap, effective, rugged, safe extraction

**RIA:** Radioimmunoassay

**RSD:** Relative standard deviation

**SBSE:** Stir-bar sorptive extraction

**SD-LPME:** Solid drop liquid phase microextraction

**SFE:** Supercritical fluid extraction

**SPE:** Solid phase extraction

**SPME:** Solid-phase microextraction

**TFME:** Thin film microextraction

**TOF:** Time of flight

**UAE:** Ultrasound-assisted extraction

**UHPLC:** Ultra-high performance liquid chromatography

**US-EPA:** Agencia de Protección del Medio Ambiente de Estados Unidos

**WADA:** World Anti-Doping Agency





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### Anexo III. Publicaciones de la Tesis Doctoral

1. **Autores:** R. Guedes Alonso, S. Montesdeoca Esponda, Z. Sosa Ferrera, J.J. Santana Rodríguez.

**Título:** Liquid chromatography methodologies for the determination of steroid hormones in aquatic environmental systems

**Revista:** Trends in Environmental Analytical Chemistry (TrEAC)

**Volumen:** 3 – 4

**Páginas:** 14 – 27

**Fecha:** 2014

2. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez.

**Título:** Optimization of a solid-phase extraction procedure combined with ultra-high performance liquid chromatography and fluorescence detection to determine estrogens in wastewater samples

**Revista:** Luminiscence

**Volumen:** 27

**Páginas:** 560-561

**Fecha:** 2012

3. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez.

**Título:** Simultaneous determination of hormonal residues in treated waters using ultrahigh performance liquid chromatography tandem mass spectrometry

**Revista:** Journal of analytical methods in chemistry

**Volumen:** ID 210653

**Páginas:** 1-8

**Fecha:** 2013

4. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez.

**Título:** An on-line solid phase extraction method coupled with UHPLC-MS/MS for the determination of steroid hormone compounds in treated water samples from wastewater treatment plants

**Revista:** Analytical methods

**Volumen:** 7

**Páginas:** 5996-6005

**Fecha:** 2015

5. **Autores:** R. Guedes Alonso, S. Santana Viera, Z. Sosa Ferrera J.J. Santana Rodríguez.

**Título:** Molecularly imprinted solid-phase extraction coupled with ultra-high performance liquid chromatography and fluorescence detection for the determination of estrogens and their metabolites in wastewater

**Revista:** Journal of Separation Science

**Volumen:** 38

**Páginas:** 3961 – 3968

**Fecha:** 2015

6. **Autores:** R. Guedes Alonso, L. Ciofi, Z. Sosa Ferrera, J.J. Santana Rodríguez, M. del Bubba, A. Kabir, K.G. Furton.

**Título:** 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

**Revista:** Journal of Chromatography A

**Volumen:** 1437

**Páginas:** 116 – 126

**Fecha:** 2016

7. **Autores:** R. Guedes Alonso, S. Santana Viera, S. Montesdeoca Esponda, C. Afonso Olivares, Z. Sosa Ferrera, J. J. Santana Rodríguez

**Título:** 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

**Revista:** Analytical and Bioanalytical Chemistry

**Volumen:** 408 (24)

**Páginas:** 6833 – 6844

**Fecha:** 2016

8. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez.

**Título:** Determination of sex hormones and glucocorticoids in fish tissues by Microwave Assisted Extraction and Solid Phase Extraction coupled to Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry

**Revista:** Food chemistry

**Volumen:** *en revisión*

**Páginas:**

**Fecha:** 2017



## Anexo IV. Comunicaciones a Congresos relacionados con la realización de la Tesis Doctoral

1. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Optimization of a solid-phase extraction procedure combined with ultra-high performance liquid chromatography and fluorescence detection to determinate estrogens in wastewater samples

**Congreso:** XV International Symposium on Luminescence Spectrometry (ISLS 2012)

**Tipo de participación:** Póster

**Lugar de celebración:** Barcelona (España)

**Fecha:** Junio de 2012

2. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Determination of steroid hormones in treated water from wastewater treatment plants of Gran Canaria (Spain) by SPE-UHPLC-MS/MS

**Congreso:** XII Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECYTA 2012)

**Tipo de participación:** Póster

**Lugar de celebración:** Tarragona (España)

**Fecha:** Noviembre de 2012

3. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Ultra-high Performance Chromatography with mass spectrometry detection (UHPLC-MS/MS) for the determination of fifteen hormonal compounds in sewage from wastewater treatment plants of Gran Canaria (Spain)

**Congreso:** 39<sup>th</sup> International Symposium on High-Performance-Liquid-Phase Separations and Related Techniques (HPLC 2013)

**Tipo de participación:** Póster

**Lugar de celebración:** Amsterdam (Países Bajos)

**Fecha:** Junio de 2013

4. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Assessment of hormonal compounds by SPE-UHPLC-MS/MS procedure in samples of wastewater treatment plants of Gran Canaria (Spain)

**Congreso:** XIII Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECYTA 2013)

**Tipo de participación:** Póster

**Lugar de celebración:** Tenerife (España)

**Fecha:** Octubre de 2013

5. **Autores:** R. Guedes Alonso, S. Montesdeoca-Esponda, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Optimization of an on-line solid phase extraction (SPE) coupled with UHPLC-MS/MS, for the determination of hormonal compounds in sewage from wastewater treatment plants of Gran Canaria (Spain)

**Congreso:** SETAC Europe 24th Annual Meeting

**Tipo de participación:** Póster

**Lugar de celebración:** Basilea (Suiza)

**Fecha:** Mayo de 2014

6. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Determination of hormones in sewage from wastewater treatment plants of Gran Canaria (Spain) using an On-line Solid Phase Extraction (SPE) coupled with Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry

**Congreso:** 10<sup>th</sup> Annual LC/MS/MS Workshop on environmental applications and food safety

**Tipo de participación:** Póster

**Lugar de celebración:** Barcelona (España)

**Fecha:** Julio de 2014

7. **Autores:** R. Guedes Alonso, S. Montesdeoca-Esponda, C. Afonso Olivares, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Optimization of a microwave assisted extraction procedure combined with ultra-high performance liquid chromatography and fluorescence detection to determine estrogens in sludge samples

**Congreso:** XVI International Symposium On Luminescence Spectrometry

**Tipo de participación:** Póster

**Lugar de celebración:** Rodas (Grecia)

**Fecha:** Septiembre de 2014

8. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Comparison of sorbents in on-line SPE coupled to UHPLC-MS/MS for the determination of hormones in water samples

**Congreso:** 14<sup>as</sup> Jornadas de Análisis Instrumental (JAI)

**Tipo de participación:** Póster

**Lugar de celebración:** Barcelona (España)

**Fecha:** Octubre de 2014

9. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Analysis of hormonal compounds in samples from wastewater treatment plants of Gran Canaria (Spain) using on-line solid phase extraction (SPE) coupled with UHPLC-MS/MS

**Congreso:** International Meeting of Environmental and Pharmaceutical Analysis (IMEPA 2014)

**Tipo de participación:** Comunicación oral

**Lugar de celebración:** Las Palmas de Gran Canaria (España)

**Fecha:** Diciembre de 2014

10. **Autores:** R. Guedes Alonso, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Determinación de hormonas esteroideas en aguas depuradas de la isla de Gran Canaria

**Congreso:** II Workshop "Estudio, aprovechamiento y gestión del agua en terrenos e islas volcánicas"

**Tipo de participación:** Comunicación oral

**Lugar de celebración:** Las Palmas de Gran Canaria (España)

**Fecha:** Enero de 2015

11. **Autores:** R. Guedes Alonso, S. Santana Viera, S. Montesdeoca-Esponda, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Optimization of molecularly imprinted solid phase extraction (MISPE) coupled with UHPLC-FD, for the determination of estrogens in wastewaters

**Congreso:** SETAC Europe 25th Annual Meeting

**Tipo de participación:** Póster

**Lugar de celebración:** Barcelona (España)

**Fecha:** Mayo de 2015

12. **Autores:** S. Santana Viera, R. Guedes Alonso, C. Afonso Olivares, S. Montesdeoca Esponda, Z. Sosa Ferrera; J.J. Santana Rodríguez

**Título:** Optimization of microwave assisted extraction combined with UHPLC with fluorescence detection for the determination of estrogens in wastewater sludge samples

**Congreso:** 42<sup>nd</sup> International Symposium on High Performance Liquid Phase Separations and Related Techniques

**Tipo de participación:** Póster

**Lugar de celebración:** Ginebra (Suiza)

**Fecha:** Junio de 2015

13. **Autores:** R. Guedes Alonso, L. Ciofi, M. del Bubba, Z. Sosa Ferrera, J.J. Santana Rodríguez, A. Kabir, K.G. Furton

**Título:** Determination of hormones in sewage by using fabric phase sorptive extraction (FPSE) and ultra-high performance liquid chromatography tandem mass spectrometry

**Congreso:** 42<sup>nd</sup> International Symposium on High Performance Liquid Phase Separations and Related Techniques

**Tipo de participación:** Póster

**Lugar de celebración:** Ginebra (Suiza)

**Fecha:** Junio de 2015

14. **Autores:** R. Guedes Alonso, S. Santana Viera, C. Afonso Olivares, S. Montesdeoca-Esponda, Z. Sosa Ferrera, J.J. Santana Rodríguez

**Título:** Analysis of hormones in sludge samples using microwave-assisted extraction and ultra-high performance liquid chromatography tandem mass spectrometry

**Congreso:** International Symposium on Chromatography (ISC2016)

**Tipo de participación:** Póster

## *Anexos*

**Lugar de celebración:** Cork (Irlanda)

**Fecha:** Agosto de 2016

15.**Autores:** R. Guedes Alonso, S. Montesdeoca-Esponda, Z. Sosa Ferrera, J.J. Santana Rodríguez

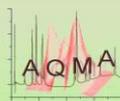
**Título:** Study of the presence of steroid steroid hormones residues in fish tissues from Gran Canaria (Spain)

**Congreso:** International Conference: Maritime Spatial Planning, Ecosystem Approach and Supporting Information Systems (MaPSIS)

**Tipo de participación:** Póster

**Lugar de celebración:** Las Palmas de Gran Canaria (España)

**Fecha:** Abril de 2017



## OPTIMIZATION OF A SOLID-PHASE EXTRACTION PROCEDURE COMBINED WITH ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION TO DETERMINE ESTROGENS IN WASTEWATER SAMPLES

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### INTRODUCTION

In recent years, the ecosystem problems, the increasing of water demand and the continuous discovery of new emerging pollutants have attracted the attention of scientific community. This is the reason why many researches have been focused on developing new methodologies to determine all substances with environmental interest.

Within this broad group of compounds, the use of estrogenic compounds has increased exponentially nowadays. (1,2).

In this research, a solid phase extraction (SPE) procedure was optimized and coupled to ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FD) for the determination of a group of four estrogens: estriol,  $\beta$ -estradiol, 17 $\beta$ -estradiol glucuronide and 17 $\alpha$ -ethinyl estradiol. All parameters involved in solid-phase extraction were optimized, such as, type of SPE cartridge, sample volume, pH and ionic strength of sample, wash step and desorption volume. The results were evaluated to obtain optimum extraction conditions.

### EXPERIMENTAL

#### Chromatographic separation and Fluorescence detection

An UHPLC system with fluorescence detector from Waters (Madrid, Spain) and a C<sub>18</sub> column were used. Analytes separation was carried out employing this gradient: starts at 55:45 (water/methanol) during 3 minutes, it changed to 50:50 (v/v) and stayed for 2.5 min. 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·min<sup>-1</sup>. Fluorescence detector was operated at an excitation and emission wavelength of 280 and 310 nm respectively.

Ident. nº	Compound	$\lambda_{ex}$	$\lambda_{em}$
1	Estriol	280 nm	310 nm
2	17 $\beta$ -estradiol glucuronide		
3	17 $\beta$ -estradiol		
4	17 $\alpha$ -ethinyl estradiol		

Table 1: Id. number of compounds and excitation and emission wavelengths

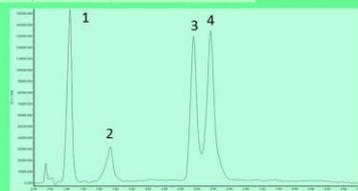


Fig. 1: Chromatogram of a spiked deionized water sample (250 ng·mL<sup>-1</sup> for each analyte) using optimized SPE method

### RESULTS

From the results obtained in the study of different cartridges, we can observe that the better signals are found for SepPak C18 cartridge (figure 2).

The pH, ionic strength and sample volume were optimized using a 2<sup>3</sup> design (three variables, two levels), because that ionic strength and sample volume present the higher correlations, a 3<sup>2</sup> experimental design was done. Figure 3 shows the response surface obtained for the estriol, where can be observe that the best conditions were 0% of NaCl and 250 mL of sample volume.

Finally, the desorption volume and the wash-step were fixed at 2 mL of methanol in one step and 5 mL of Milli-Q water without methanol respectively. In these conditions we achieved a preconcentration factor of 125.

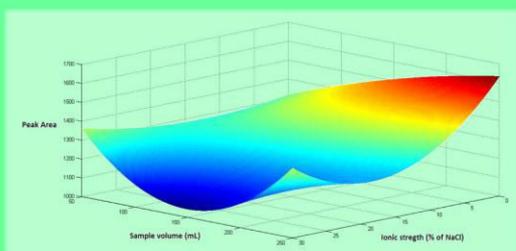


Fig. 3: Effect of ionic strength and sample volume on the SPE extraction of Estriol.

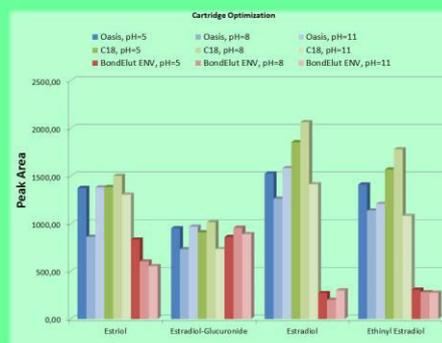


Fig. 2: Optimization of SPE cartridges at different pH.

### ACKNOWLEDGEMENT

This work was supported by funds provided by the Spanish Ministry of Science and Innovation (MICINN) under research project reference CTQ 2010-20554



### CONCLUSIONS

In accordance with the obtained results, the optimum conditions for SPE procedure were: SepPak C18 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.

### REFERENCES

- Falconer, I.R; Chapman, H.F; Moore, M.R; Ranmuthugala, G. 2006. Environmental Toxicology, 21(2): 181-191.
- Vega-Morales, T; Sosa-Ferrera, Z; Santana-Rodríguez, J.J. 2010. Journal of Hazardous Materials, 183: 701-711.





OPTIMIZATION OF A SOLID-PHASE EXTRACTION PROCEDURE COMBINED WITH ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION TO DETERMINE ESTROGENS IN WASTEWATER SAMPLES

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**INTRODUCTION**

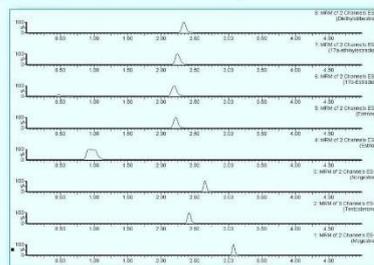
The use of hormones has increased exponentially in the last 50 years. Because of that, its concentration in wastewater begins to be a significant problem in Wastewater Treatment Plants. (WWTPs) This increase of concentrations is due to the hormones metabolization only in a small percentage, as well as the continuous introduction of them into the environment. [1,2] In this research, a solid phase extraction (SPE) coupled with ultra-high performance liquid chromatography (UHPLC) procedure was optimized for the determination of a group of eight steroid hormones, estriol, 17β-estradiol, 17α-ethinyl estradiol, estrone, diethylstilbestrol, testosterone, levonorgestrel and megestrol acetate in water samples All parameters involved in solid-phase extraction and chromatographic detection were optimized, and the method was applied to different wastewater samples.

**EXPERIMENTAL**  
**SPE optimization**

Optimized variables	
Cartridge	Sep-Pak C18
pH and ionic strength	pH=8, 0% NaCl
Sample volume	250 ml
Wash step	5 ml. of Milli-Q water
Desorption volume	2ml. of Methanol
Preconcentration factor	125

Table 1. Optimized variables for SPE procedure. [3]

**UHPLC-MS/MS conditions**



Minute	% of MeOH
0:00	50
3:00	75
4:00	50
5:30	50

Table 2. Gradient used in UHPLC-MS/MS

Figure 1. Chromatogram (MRM) obtained from UHPLC-MS/MS

**RESULTS**

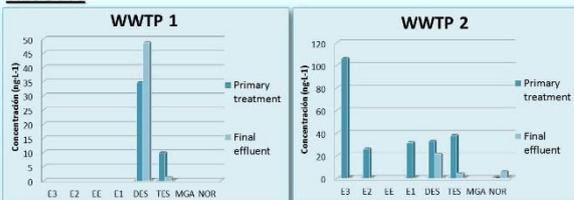


Figure 2. Concentrations obtained in both WWTP for the eight hormones studied

Compound	Analytical parameters			
	LOD <sup>a</sup> (ng·L <sup>-1</sup> )	LOQ <sup>b</sup> (ng·L <sup>-1</sup> )	RSD <sup>c</sup> (%)	Recovery (%)
Estriol (E3)	9.35	31.18	6.5	80.5 ± 5.3
17β-estradiol (E2)	2.53	8.44	8.4	89.7 ± 7.5
17α-ethinylestradiol (EE)	0.51	1.71	7.3	90.6 ± 6.5
Estrone (E1)	2.60	8.66	6.8	78.7 ± 5.4
Diethylstilbestrol (DES)	0.64	2.14	6.9	50.7 ± 3.5
Testosterone (TES)	1.49	4.95	6.8	83.8 ± 5.7
Megestrol acetate (MGA)	0.15	0.49	7.2	73.7 ± 5.3
Norgestrel (NOR)	2.11	7.04	7.4	88.9 ± 6.5

<sup>a</sup> Detection Limits <sup>b</sup> Quantification Limits <sup>c</sup> Relative Standard Deviation

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

The method developed was applied to samples of two wastewater treatment plants with different water treatments. WWTP 1 uses a traditional method based on Activate sludge, while WWTP 2 presents a Bioreactor membrane treatment. In WWTP 2 were detected more compounds than WWTP 1, and in WWTP 2, the concentrations were lower in effluent (except Norgestrel), so bioreactor membrane treatment may be effective for the studied compounds. For WWTP 1, the results are not conclusive, because only were detected two of eight hormones under study.

**CONCLUSIONS**

In accordance with the obtained results, the SPE-UHPLC-MS/MS procedure optimized was easy, cheap, selective and sensitive, with low detection and quantification limits. The application in samples from WWTPs was satisfactory and these samples showed appreciable amounts of the compounds under study.

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1. R.P. Schwarzenbach, B.I. Escher, K. Fenner, T.B. Hofstetter, C.A. Johnson, U. von Gunten, B. Wehrli. Science. 313 (2006) 1072-1077.
2. T. Vega-Morales, Z. Sosa-Ferrera, J.J. Santana-Rodríguez. Journal of Hazardous Materials. 183 (2010) 701-711.
3. R. Guedes-Alonso, Z. Sosa-Ferrera, J.J. Santana-Rodríguez. Luminiscence. DOI 10.1002/bio.2432

**ACKNOWLEDGEMENT**

This work was supported by funds provided by the Spanish Ministry of Science and Innovation (MICINN) under research project reference CTQ 2010-20554

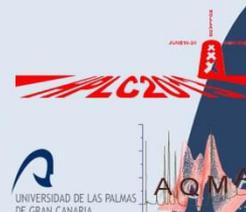


XII Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECYTA 2012)

Tarragona (España), noviembre de 2012



# ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH MASS SPECTROMETRY DETECTION (UHPLC-MS/MS) FOR THE DETERMINATION OF FIFTEEN HORMONAL COMPOUNDS IN WASTEWATER FROM WASTEWATER TREATMENT PLANTS OF GRAN CANARIA (SPAIN)



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## INTRODUCTION:

From all of emerging pollutants, the hormonal residues constitute a group of great interest due to they are considered as endocrine disruptor 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 [1]. The hormones are used in human and veterinary medicine, and their consumption has increased exponentially, which produces a continuous introduction of them into the environment [2].

In this study, the determination of a group of fifteen natural and synthetic hormones (five estrogens, three androgens, four progestogens and three corticosteroids), is presented. The extraction and preconcentration method chosen has been Solid Phase Extraction (SPE) which has been optimized and combined with ultra-high performance liquid chromatography coupled to mass spectrometry detection (UHPLC-MS/MS). The hormonal compounds have been studied in wastewater samples from wastewater treatment plants in Gran Canaria (Spain), with different water treatment methods.

## EXPERIMENTAL PROCEDURE:

### Chromatographic conditions:

Instrument	Waters ACQUITY UPLC with TQ Detector
Column	ACQUITY UHPLC BEH Waters C <sub>18</sub>
Injection volume	10 µL
Flow rate	0.3 mL·min <sup>-1</sup>
Mobil phases	A: Water (0.1% v/v NH <sub>3</sub> + 15 mM CH <sub>3</sub> COONH <sub>4</sub> ) B: Methanol

### Gradient used:

Time (min)	% (A)	% (B)
0.00	80	20
1.50	40	60
2.75	25	75
3.75	25	75
6.00	80	20
6.50	80	20

### Optimum SPE conditions:

Cartridge	SepPak C <sub>18</sub> 6cc (Waters)
Sample volume	250 mL
Sample pH	8
Ionic strength	0% NaCl
Wash step	5 mL Milli-Q water
Desorption solvent	Methanol
Desorption volume	2 mL (1 mL·min <sup>-1</sup> )

## RESULTS:

### Analytical parameters:

Compound	RT <sup>a</sup> (min)		LOD <sup>b</sup> (ng·L <sup>-1</sup> )		RSD <sup>c</sup> (%)		Recovery (%)	
	(min)	(min)	(25 ng·L <sup>-1</sup> ) n=6	(150 ng·L <sup>-1</sup> ) n=6	(25 ng·L <sup>-1</sup> ) n=3	(150 ng·L <sup>-1</sup> ) n=3	(25 ng·L <sup>-1</sup> ) n=3	(150 ng·L <sup>-1</sup> ) n=3
Estril	2.12	1.35	5.12	6.50	106.6 ± 8.2	99.0 ± 1.8		
Prednisone	2.29	0.27	12.9	4.79	121.3 ± 5.3	105.0 ± 3.3		
Cortisone	2.32	0.05	4.55	4.50	118.5 ± 1.2	103.2 ± 6.3		
Prednisolone	2.45	0.18	6.90	4.56	112.9 ± 5.6	99.2 ± 3.1		
Boldenone	2.87	0.17	5.67	4.47	117.2 ± 3.5	112.7 ± 10.8		
Nandrolone	2.93	1.00	3.83	3.81	119.4 ± 4.6	102.2 ± 5.7		
Norethisterone	2.95	0.22	7.08	4.37	113.0 ± 1.8	99.3 ± 5.1		
17β-estradiol	2.97	1.63	12.4	6.57	120.4 ± 8.5	103.0 ± 7.8		
Estrone	2.99	0.35	3.51	4.87	122.2 ± 9.9	109.3 ± 2.5		
17α-ethinylestradiol	3.00	2.95	6.84	6.63	--	103.0 ± 10.6		
Diethylstilbestrol	3.02	0.16	9.15	5.79	98.8 ± 14.2	80.4 ± 12.0		
Testosterone	3.15	0.04	6.33	3.82	118.7 ± 3.3	103.3 ± 5.4		
Norgestrel	3.29	0.32	5.14	3.68	114.9 ± 2.5	103.4 ± 4.4		
Megestrol acetate	3.54	0.22	8.69	4.98	107.4 ± 1.2	99.4 ± 3.4		
Progesterone	3.71	0.21	7.41	4.59	115.9 ± 4.0	100.6 ± 4.3		

<sup>a</sup> Retention time

<sup>b</sup> Detection limit

<sup>c</sup> Relative Standard Deviation

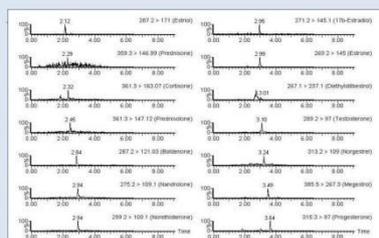


Figure 1: Chromatogram of spiked wastewater sample (200 ng·L<sup>-1</sup> of each analyte) using SPE-UHPLC method

- Wastewater samples were collected in two WWTPs located in Gran Canaria (Spain)
- Samples from both WWTPs were taken from the influents and effluents of the plants, but only in influent samples hormones were detected.

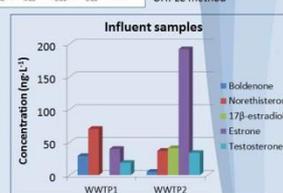


Figure 2: Concentrations of hormones detected in influent samples.

## CONCLUSIONS:

An analytical method for the simultaneous extraction, preconcentration and determination of fifteen hormones in wastewater matrices has been optimized and developed. The limits of detection reached were between 0.04 – 1.63 ng·L<sup>-1</sup>. 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·L<sup>-1</sup>.

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## ACKNOWLEDGEMENTS:

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

39<sup>th</sup> International Symposium on High-Performance-Liquid-Phase Separations and Related Techniques (HPLC 2013)

Amsterdam (Países Bajos), junio de 2013



## ASSESSMENT OF HORMONAL COMPOUNDS BY SPE-UHPLC-MS/MS PROCEDURE IN SAMPLES OF WASTEWATER TREATMENT PLANTS OF GRAN CANARIA (SPAIN)

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### INTRODUCTION

Determination and control of emerging pollutants have been important topics in environmental analytical chemistry in the last decades. 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 [1]. The consumption of hormones has been increased exponentially in the last decades due to the use of hormones in human and veterinary medicine. Since 1990s some studies established that the main pathway of these compounds to go into the environment is through effluent of wastewater treatment plants (WWTPs). Because of that, many studies in the last years determine the concentrations of sexual hormones in water and wastewater samples [2,3].

In this study, the determination of a group of several natural and synthetic hormones (estrogens, androgens, progestogens and corticosteroids), is presented. For their determination a method based on Solid Phase Extraction (SPE) combined with ultra-high performance liquid chromatography coupled to mass spectrometry detection (UHPLC-MS/MS) have been chosen.

The hormonal compounds have been studied in wastewater samples from four wastewater treatment plants around Gran Canaria island (Spain), with different water treatment methods (WWTP<sub>1</sub> has a membrane bioreactor, WWTP<sub>2</sub> and 4 use activated sludge technology and WWTP<sub>3</sub> uses natural treatment methods) to evaluate the presence of this type of compounds in different settlements of the island.

### EXPERIMENTAL PROCEDURE

#### Chromatographic conditions

Instrument	Waters ACQUITY UPLC with TQ Detector
Column	ACQUITY UHPLC BEH Waters C <sub>18</sub>
Injection volume	10 µL
Flow rate	0.3 mL·min <sup>-1</sup>
Mobil phases	A: Water (0.1% v/v NH <sub>3</sub> ) B: Methanol

Time (min)	% (A)	% (B)
0.00	80	20
1.50	40	60
2.75	25	75
3.75	25	75
6.00	80	20
6.50	80	20

#### Gradient used

#### Optimum SPE conditions

Cartridge	SepPak C <sub>18</sub> 6cc (Waters)
Sample volume	250 mL
Sample pH	8
Ionic strength	0% NaCl
Wash step	5 mL Milli-Q water
Desorption solvent	Methanol
Desorption volume	2 mL (1 mL·min <sup>-1</sup> )
Cartridge	SepPak C <sub>18</sub> 6cc (Waters)

### RESULTS

#### Analytical parameters

Compound	RT <sup>a</sup>	LOD <sup>b</sup>	RSD <sup>c</sup> (%)		Recovery (%)	
	(min)	(ng·L <sup>-1</sup> )	(25 ng·L <sup>-1</sup> ) n=6	(150 ng·L <sup>-1</sup> ) n=6	(25 ng·L <sup>-1</sup> ) n=3	(150 ng·L <sup>-1</sup> ) n=3
Estriol	2.12	1.35	5.12	6.50	106.6 ± 8.2	99.0 ± 1.8
Prednisone	2.29	0.27	12.9	4.79	121.3 ± 5.3	105.0 ± 3.3
Cortisone	2.32	0.05	4.55	4.50	118.5 ± 1.2	103.2 ± 6.3
Prednisolone	2.45	0.18	6.90	4.56	112.9 ± 5.6	99.2 ± 3.1
Boldenone	2.87	0.17	5.67	4.47	117.2 ± 3.5	112.7 ± 10.8
Nandrolone	2.93	1.00	3.83	3.81	119.4 ± 4.6	102.2 ± 5.7
Norethisterone	2.95	0.22	7.08	4.37	113.0 ± 1.8	99.3 ± 5.1
17β-estradiol	2.97	1.63	12.4	6.57	120.4 ± 8.5	103.0 ± 7.8
Estrone	2.99	0.35	3.51	4.87	122.2 ± 9.9	109.3 ± 2.5
17α-ethinylestradiol	3.00	2.95	6.84	6.63	--	103.0 ± 10.6
Diethylstilbestrol	3.02	0.16	9.15	5.79	98.8 ± 14.2	80.4 ± 12.0
Testosterone	3.15	0.04	6.33	3.82	118.7 ± 3.3	103.3 ± 5.4
Norgestrel	3.29	0.32	5.14	3.68	114.9 ± 2.5	103.4 ± 4.4
Megestrol acetate	3.54	0.22	8.69	4.98	107.4 ± 1.2	99.4 ± 3.4

<sup>a</sup> Retention time <sup>b</sup> Detection limit <sup>c</sup> Relative Standard Deviation

#### Real samples

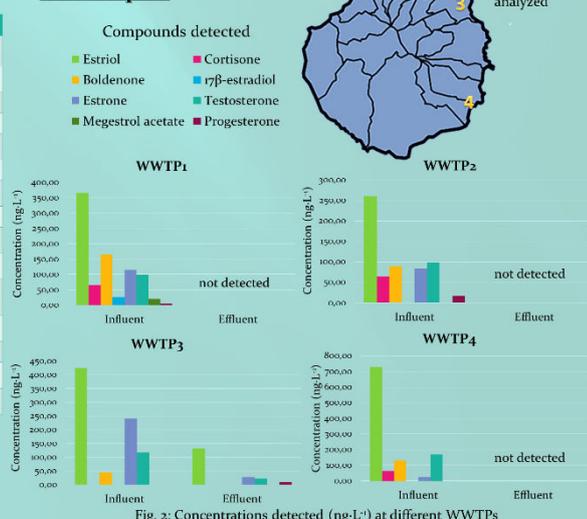


Fig. 2: Concentrations detected (ng·L<sup>-1</sup>) at different WWTPs

### CONCLUSIONS

An analytical method for the simultaneous extraction, preconcentration and determination of fifteen hormones in wastewater matrices has been optimized and developed, obtained high recoveries and RSD lower than 13%. The application of the method to samples from two different WWTPs showed that eight hormones were detected at the different WWTPs, ranging from few ng·L<sup>-1</sup> to various cents of ng·L<sup>-1</sup>. Hormones were detected only in influent samples of three WWTPs, and only in WWTP<sub>3</sub>, hormones were detected in influent and effluent samples.

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### ACKNOWLEDGEMENTS

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XIII Scientific Meeting of the Spanish Society of Chromatography and Related Techniques (SECYTA 2013)

Tenerife (España), octubre de 2013



# Optimization of an on-line solid phase extraction (SPE) coupled with UHPLC-MS/MS, for the determination of hormonal compounds in sewage from wastewater treatment plants of Gran Canaria (Spain)

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## INTRODUCTION:

Steroid hormones are an important group inside of the emerging pollutants, and are considered as endocrine disruptor compounds (EDCs) due to their capacity of altering the natural hormonal equilibrium, producing harmful effects in animals, humans and their progeny. These changes are more noticeable in the marine environment [1]. The consumption of steroid hormones has increased exponentially in last decades, due to their use in human and veterinary medicine and the principal source of these pollutants are the wastewater treatment plants. Several studies have determined the presence of this type of compounds in wastewater samples [2].

An on-line solid phase extraction process coupled with ultra-high performance liquid chromatography following by mass spectrometry detection (UHPLC-MS/MS) has been optimized to determine fourteen natural and synthetic hormones (Table 1).

## EXPERIMENTAL:

### Compounds studied:

Estrogens	Androgens	Progestogens	Corticosteroids
Diethylstilbestrol (DES)	Boldenone (BOL)	Norethisterone (NOR)	Prednisone (PRD)
17β-estradiol (E2)	Nandrolone (NAN)	Norgestrel (NRG)	Cortisone (COR)
Estrone (E1)	Testosterone (TES)	Megestrol Acetate (MGA)	Prednisolone (PRDL)
Estriol (E3)		Progesterone (PRO)	

Table 1: Compounds studied

### Extraction and chromatographic conditions:

Time (min)	BSM				QSM			
	Flow (mL·min <sup>-1</sup> )	A (%)	B (%)	Flow (mL·min <sup>-1</sup> )	A2 (%)	B2 (%)	C (%)	D (%)
0.00	0.300	80	20	2.000	100	0	0	0
0.50	0.300	80	20	2.000	0	100	0	0
3.80	0.300	80	20	2.000	0	100	0	0
4.10	0.300	80	20	2.000	0	0	0	100
7.00	0.300	0	100	2.000	100	0	0	0
8.00	0.300	0	100	2.000	100	0	0	0
10.50	0.300	80	20	2.000	100	0	0	0

• Column: ACQUITY UPLC BEH Waters C18 (50 x 2.1 mm, 1.7 μm)  
• Mobile phases: A: Water + 0.1% NH<sub>3</sub> and B: Methanol

→ Loading phase  
→ Weak wash step  
→ Strong wash of the cartridges  
→ Re-equilibration time

Table 2: Gradient used for extraction process and chromatographic separation

## On-line SPE optimization:

### i) Cartridge used

Two Oasis HLB columns (20 μm, 2.1x30mm) working in parallel.  
Flow rate: 2 mL·min<sup>-1</sup>.  
Mobile phases consist of:  
A2: water (0.05% acetic acid)  
B2: water without additives  
C: methanol without additives  
D: methanol:acetone:hexane

### iii) Load phase and sample volume

**Load phases studied:**  
→ pH = 3.4 (Water + 0.05% CH<sub>3</sub>COOH)  
→ pH = 5.8 (Water)  
→ pH = 8.1 (Water + 0.1% NH<sub>3</sub> + 0.1M CH<sub>3</sub>COONH<sub>4</sub>)  
→ pH = 10.1 (Water 0.1% NH<sub>3</sub>)

**Sample volume:** 1, 2, 3, 4 and 5 mL of wastewater

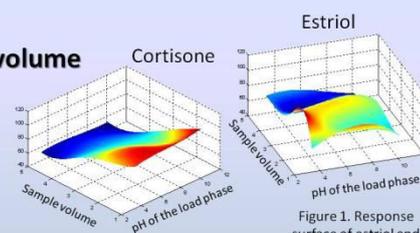


Figure 1. Response surface of estriol and cortisone in load phase and sample volume optimization

### ii) pH of the sample

**pH studied:**  
→ pH = 3.5 (using acetic acid)  
→ pH = 5.7 (without additives)  
→ pH = 10.4 (using NH<sub>3</sub>)

### iv) Wash step

**Solvents studied:**  
→ Water and MeOH without additives  
→ Water and MeOH with 0.1% NH<sub>3</sub>

**% of organic solvent:**  
→ 0% of MeOH  
→ 10% of MeOH  
→ 20% of MeOH  
→ 30% of MeOH  
→ 40% of MeOH

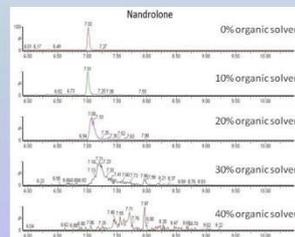


Figure 2. Chromatographic peaks of nandrolone with different proportions of water:methanol in the wash step optimization

## RESULTS:

### Analytical parameters:

Compound	Detection limit (ng·L <sup>-1</sup> )	100 ng·L <sup>-1</sup>		500 ng·L <sup>-1</sup>	
		Recovery (%) n=6	RSD* (%) n=6	Recovery (%) n=6	RSD* (%) n=6
Diethylstilbestrol	13.2	44.3	7.3	42.3	14.7
17β-estradiol	8.5	88.8	26.4	104.0	7.0
Estrone	4.1	75.1	15.1	81.6	8.8
Estriol	4.5	76.8	5.2	69.7	17.1
Norgestrel	1.6	34.5	8.6	36.7	11.6
Testosterone	1.0	43.1	6.9	48.3	3.7
Megestrol acet.	1.2	138.7	6.8	154.4	10.8
Prednisone	9.2	61.7	11.5	60.7	5.0
Prednisolone	6.1	95.2	9.4	100.0	8.7
Cortisone	2.1	69.5	7.3	66.3	3.2
Boldenone	0.7	61.1	4.5	67.5	2.7
Norethisterone	2.3	42.7	2.9	44.3	3.3
Nandrolone	4.1	59.0	9.6	59.6	3.3
Progesterone	0.5	43.4	10.7	43.7	10.3

Table 3. Analytical parameters of the method. \* Relative standard deviation

### Real samples:

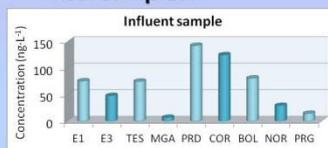


Figure 3. Concentrations detected in influent sample

Wastewater samples were collected from a WWTP located in Gran Canaria (Spain) WWTP had a novel membrane bioreactor system (MBR). The samples were taken from the influent of the plant.

## CONCLUSIONS

In accordance with the obtained results, the on-line SPE-UHPLC-MS/MS procedure is easy, cheap, selective and sensitive, with low detection and quantification limits. The application in real samples from WWTPs was satisfactory.

## REFERENCES

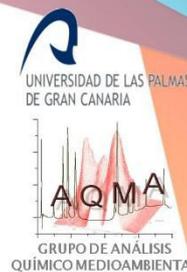
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# Determination of hormones in sewage from wastewater treatment plants of Gran Canaria (Spain) using an On-line Solid Phase Extraction (SPE) coupled with Ultra-High Performance Liquid Chromatography tandem Mass Spectrometry

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## INTRODUCTION

Steroid hormones are an important group considered as endocrine disruptor compounds (EDCs) and their consumption has increased exponentially in last decades, due to their use in human and veterinary medicine. The introduction of hormones into the environment is continuous because the principal source of these pollutants is the wastewater treatment plants [1]. For this reason hormones can produce harmful effects into the environment, especially into the aquatic environment.

An on-line solid phase extraction process coupled with ultra-high performance liquid chromatography following by tandem mass spectrometry detection (UHPLC-MS/MS) has been optimized and applied to determine fourteen natural and synthetic hormones of different types (Table 1).

## EXPERIMENTAL:

### Compounds studied:

Estrogens:	Diethylstilbestrol (DES)	17β-estradiol (E2)	Estrone (E1)	Estriol (E3)
Progestogens:	Norethisterone (NORET)	Megestrol Acetate (MGA)	Norgestrel (NRG)	Progesterone (PRG)
Androgens:	Testosterone (TES)	Nandrolone (NAN)	Boldenone (BOL)	
Glucorticoids:	Prednisolone (PRDL)	Prednisone (PRD)	Cortisone (COR)	

Table 1. Compounds studied

### Chromatographic and extraction conditions:

Time (min)	BSM				QSM				
	Flow (mL·min <sup>-1</sup> )	A (%)	B (%)	Flow (mL·min <sup>-1</sup> )	A2 (%)	B2 (%)	C (%)	D (%)	
0.00	0.300	80	20	2.000	100	0	0	0	Loading phase
0.50	0.300	80	20	2.000	0	100	0	0	Weak wash step
3.80	0.300	80	20	2.000	0	100	0	0	
4.10	0.300	80	20	2.000	0	0	0	100	Strong wash step
7.00	0.300	0	100	2.000	100	0	0	0	Re-equilibration time
8.00	0.300	0	100	2.000	100	0	0	0	
10.50	0.300	80	20	2.000	100	0	0	0	

Table 2. Extraction conditions and chromatographic gradient

- Column: ACQUITY UPLC BEH Waters C18 (50 x 2.1 mm, 1.7 μm)
- Mobile phases: A: Water + 0.1% NH<sub>3</sub> and B: Methanol  
A2: Water + 0.05% acetic acid  
B2 & C: Water and Methanol without additives  
D: Mixture 1:1:1 of methanol:acetone:hexane

### Extraction and detection parameters:

An Acquity UPLC system was used. It consists in a quaternary pump for the extraction process, a binary pump for the chromatographic separation and a triple quadrupole detector (TQD)



Acquity UPLC system

### On-line SPE conditions:

- SPE sorbent: Two Oasis HLB columns (20 μm, 2.1x30mm) working in parallel.
- Sample volume: 2000 mL of filtered by 0.22 μm wastewater
- Sample pH: Basic pH (pH ≈ 10)



Oasis HLB columns

### MS/MS detection conditions:

- Capillary voltage: 3.5 kV (ESI+) and -2.5 kV (ESI-)
- Source temperature: 150°C
- Desolvation temperature: 500°C
- Desolvation gas: Nitrogen at 1000 L·hr<sup>-1</sup>
- Collision gas: Argon at 0.15 mL·min<sup>-1</sup>

## RESULTS:

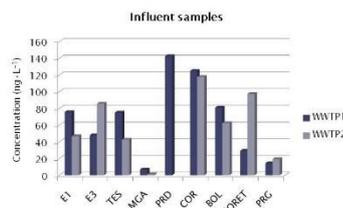
### Analytical parameters

Compound	Detection limit (ng·L <sup>-1</sup> )	100 ng·L <sup>-1</sup>		500 ng·L <sup>-1</sup>	
		Recovery (%)	RSD* (%)	Recovery (%)	RSD* (%)
Diethylstilbestrol	13.2	44.3	7.3	42.3	14.7
17β-estradiol	8.5	88.8	26.4	104.0	7.0
Estrone	4.1	75.1	15.1	81.6	8.8
Estriol	4.5	76.8	5.2	69.7	17.1
Norgestrel	1.6	34.5	8.6	36.7	11.6
Testosterone	1.0	43.1	6.9	48.3	3.7
Megestrol acet.	1.2	138.7	6.8	154.4	10.8
Prednisone	9.2	61.7	11.5	60.7	5.0
Prednisolone	6.1	95.2	9.4	100.0	8.7
Cortisone	2.1	69.5	7.3	66.3	3.2
Boldenone	0.7	61.1	4.5	67.5	2.7
Norethisterone	2.3	42.7	2.9	44.3	3.3
Nandrolone	4.1	59.0	9.6	59.6	3.3
Progesterone	0.5	43.4	10.7	43.7	10.3

Table 3. Analytical parameters \* Relative standard deviation

### Real samples

Sewage samples were collected from two WWTPs located in Gran Canaria (Spain). WWTP1 presents a membrane bioreactor technique while WWTP2 has a treatment with activated sludge



Concentrations determined in influent samples of both WWTPs

## CONCLUSIONS

In accordance with the obtained results, the on-line SPE-UHPLC-MS/MS procedure is easy, selective and sensitive, with low detection and quantification limits. The application in real samples from WWTPs was satisfactory.

## REFERENCES

- [1] T. Vega-Morales, Z. Sosa-Ferrera, J.J. Santana-Rodríguez. J. Hazard. Mater. 183 (2010) 701-711.



## OPTIMIZATION OF A MICROWAVE ASSISTED EXTRACTION PROCEDURE COMBINED WITH ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION TO DETERMINE ESTROGENS IN SLUDGE SAMPLES

Rayco Guedes-Alonso, Sarah Montesdeoca-Esponda, Cristina Afonso-Olivares, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez

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### Introduction

Endocrine disrupting compounds (EDCs) are a group of natural and synthetic compounds that worries the international community because they interfere the normal activities of the human and animal endocrine systems. Estrogens are female-steroid hormones and one of the groups of EDCs because these compounds could alter normal reproductive or development of organisms and produce feminization of fish when are discharged into the environment [1].

Estrogens are lipophilic compounds, so they tend to accumulate in solid matrices as sewage sludges. Many studies have reported the presence of estrogens in wastewater sludge samples [2].

A microwave assisted extraction (MAE) procedure is optimized and coupled to ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FD) for the determination of a group of five estrogens. All parameters involved in MAE are optimized and the results are evaluated to use optimum extraction conditions.

### Materials and methods

#### Chromatographic separation

- Waters Acquity UHPLC system with fluorescence detector
- Excitation wavelength: 280 nm
- Emission wavelength: 310 nm
- Gradient used:

Time (min)	Flow (ml·min <sup>-1</sup> )	% A	% B
0.00	0.3	80	20
1.50	0.3	50	50
2.80	0.3	50	50
3.80	0.3	0	100
6.00	0.3	80	20

A: Water + 0.1% NH<sub>3</sub> B: Acetonitrile



#### Microwave assisted extraction



- Parameters studied:
  - Power: 100, 200 and 300 W
  - Extraction Time: 2, 5 and 10 min
  - Solvent volume: 3, 6 and 9 mL of ACN

### Results

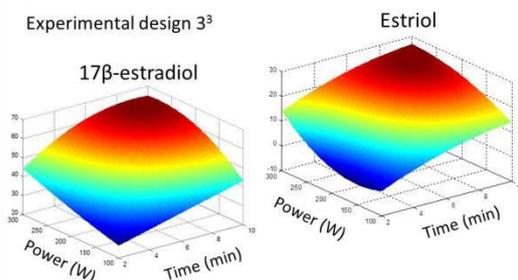


Figure 1. Surface responses of estriol and 17β-estradiol for the optimization of power and time

Optimum extraction conditions:

- Power: 300 W
- Time: 10 min
- Extraction volume: 9 mL of ACN

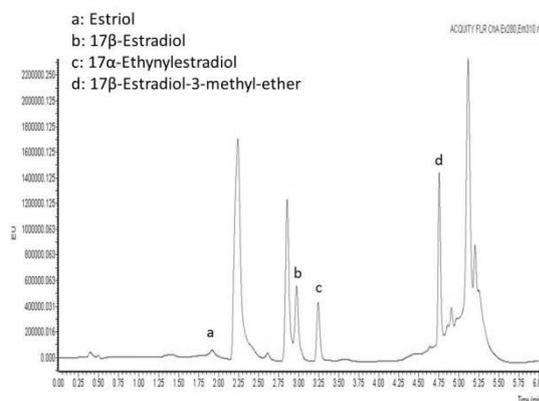


Figure 2. Chromatogram of sludge sample spiked with the estrogens mixture

### Conclusions

It has been optimized the conditions to the selective determination of the four estrogens in sludge samples by using UHPLC-FD.

The optimum extraction is done at 300 W of power, in 10 min and with 9 mL of acetonitrile as extractant

### References

- [1] H. Hamid, C. Eskicioglu. *Water Research*, **46** (2012) 5813-5833
- [2] T. Vega-Morales, Z. Sosa-Ferrera, J.J. Santana-Rodríguez. *Talanta*, **85** (2011) 1825-1834

### Acknowledgements

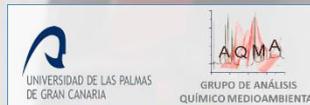
Rayco Guedes-Alonso thanks the University of Las Palmas de Gran Canaria (Spain) for his Ph.D. student grant.



## COMPARISON OF SORBENTS IN ON-LINE SPE COUPLED TO UHPLC-MS/MS FOR THE DETERMINATION OF HORMONES IN WATER SAMPLES

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### INTRODUCTION

Endocrine disrupting compounds (EDCs) are a wide group of compounds that can affect the endocrine system of organisms, producing different problems, like changes in fertility or feminization [1]. These changes are linked with the effluent discharges of wastewater treatment plants, because these are the principal way of EDCs into the environment. Steroid hormones have been measured in environmental waters at very low concentrations, so it is necessary to develop extraction and preconcentration methodologies to achieve these concentrations. Solid phase extraction (SPE) has been used widely in recent years to separate and preconcentrate hormones from environmental water samples [2] and the use of on-line SPE presents many advantages like less sample handling or shorter time analysis.

In this study, a optimization of two different sorbents used in on-line SPE coupled with ultra-high performance liquid chromatography following by tandem mass spectrometry detection (on line SPE-UHPLC-MS/MS) has been developed to determine several natural and synthetic hormones (estrogens, androgens, progesterones and corticosteroids) in effluent wastewater samples.

### EXPERIMENTAL

#### Compounds studied:

<b>Estrogens:</b>	Diethylstilbestrol	17 $\beta$ -estradiol	Estrone	Estriol
<b>Progesterogens:</b>	Norethisterone	Megestrol Acetate	Norgestrel	Progesterone
<b>Androgens:</b>	Testosterone	Nandrolone	Boldenone	
<b>Glucorticoids:</b>	Prednisolone	Prednisone	Cortisone	

#### Extraction conditions and gradient used:

Time (min)	Binary Solvent Manager				Quaternary Solvent Manager													
	Flow (mL·min <sup>-1</sup> )	A1 B1		Flow (mL·min <sup>-1</sup> )		A2 B2 C D												
		(%)	(%)	OASIS HLB	XBridge C18	(%)	(%)	(%)	(%)									
0.00	0.300	80	20	2.000	2.000	100	0	0	0	0	0	0	0	0	0	0	0	0
0.50	0.300	80	20	2.000	0.010	0	100	0	0	0	0	0	0	0	0	0	0	0
3.80	0.300	80	20	2.000	0.010	0	100	0	0	0	0	0	0	0	0	0	0	0
4.10	0.300	80	20	2.000	2.000	0	0	0	0	100	0	0	0	0	0	0	0	0
7.00	0.300	0	100	2.000	2.000	100	0	0	0	0	0	0	0	0	0	0	0	0
8.00	0.300	0	100	2.000	2.000	100	0	0	0	0	0	0	0	0	0	0	0	0
10.50	0.300	80	20	2.000	2.000	100	0	0	0	0	0	0	0	0	0	0	0	0

- Column: ACQUITY UPLC BEH Waters C18 (50 x 2.1 mm, 1.7  $\mu$ m)
- Mobile phases: A1: Water + 0.1% NH<sub>3</sub> and B1: Methanol

#### On-line SPE optimization:



On-line SPE columns

- **SPE columns:**
  - Oasis HLB (20  $\mu$ m, 2.1x30mm)
  - XBridge C<sub>18</sub> (10  $\mu$ m, 2.1x30mm)
- **Sample volume:** 1000 to 5000 of wastewater filtered by 0.22  $\mu$ m
- **Load phase:**
  - pH = 3.4 (Water + 0.05% CH<sub>3</sub>COOH)
  - pH = 5.8 (Water)
  - pH = 10.1 (Water 0.1% NH<sub>3</sub>)
- **Wash step:**
  - Water without additives
  - Water with 0.1% NH<sub>3</sub> + Different concentrations of methanol
- **Sample pH:**
  - pH = 3.5
  - pH = 5.5 - 7
  - pH = 10.4

### RESULTS

#### Optimum extraction conditions

#### OASIS HLB column:

- **Sample volume:** 2000  $\mu$ L of wastewater
- **Load phase:** pH = 3.4 (Water + 0.05% CH<sub>3</sub>COOH)
- **Wash step:** Water without additives
- **Sample pH:** pH = 10.4

#### XBridge C<sub>18</sub>

- **Sample volume:** 2000  $\mu$ L of wastewater
- **Load phase:** pH = 10.4 (Water + 0.1% NH<sub>3</sub>)
- **Wash step:** No wash step
- **Sample pH:** pH = 10.4

#### Recoveries:

- **Estrogens:** 42 – 104%
- **Progesterogens:** 48 – 68%
- **Androgens:** 36 – 150%
- **Glucorticoids:** 60 – 100%

#### Recoveries:

- **Estrogens:** < 15%
- **Progesterogens:** 9 – 57%
- **Androgens:** 11 – 26%
- **Glucorticoids:** 13%
- Estriol, prednisone and prednisolone are not extracted with these cartridges

### CONCLUSIONS

The optimization of two different sorbents used in on-line SPE coupled to UHPLC-MS/MS has been studied. OASIS SPE columns are better for the extraction of steroid hormones of wastewater with acceptable recoveries that ranged from 40 to 100% for the most of compounds under study. XBridge SPE columns are not so efficient for the extraction. In fact, some analytes as estriol, prednisone and prednisolone are not extracted with this type of cartridges. The recoveries for XBridge columns are below 40% for the most of compounds.

### REFERENCES

- [1] C. Wang, R.P. Croll. *Aquaculture* **238** (2004) 483–498
- [2] R. Guedes-Alonso, Z. Sosa-Ferrera, J. J. Santana-Rodríguez. *Journal of Analytical Methods in Chemistry* **Article ID 210653** (2014) 1–8.

### ACKNOWLEDGEMENTS

Rayco Guedes-Alonso thanks the University of Las Palmas de Gran Canaria (Spain) for his Ph.D. student grant.

14<sup>as</sup> Jornadas de Análisis Instrumental (JAI)

Barcelona (España), octubre de 2014



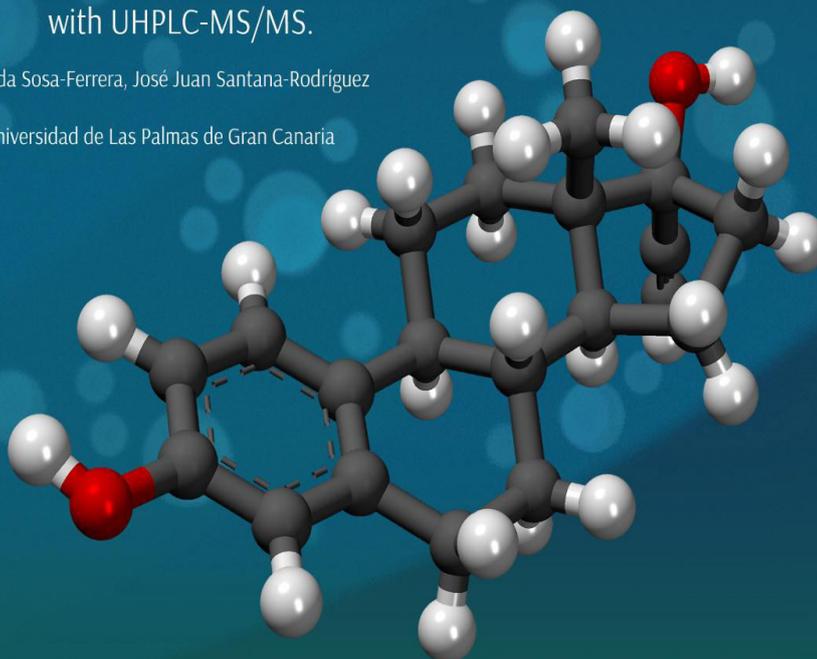


Analysis of hormonal compounds in samples from  
wastewater treatment plants of Gran Canaria (Spain)  
using on-line solid phase extraction (SPE) coupled  
with UHPLC-MS/MS.



Rayco Guedes-Alonso, Zoraida Sosa-Ferrera, José Juan Santana-Rodríguez

Departamento de Química. Universidad de Las Palmas de Gran Canaria



International Meeting of Environmental and Pharmaceutical Analysis  
(IMEPA 2014)

Las Palmas de Gran Canaria (España), diciembre de 2014







**DETERMINACIÓN DE  
HORMONAS  
ESTEROIDEAS EN AGUAS  
DEPURADAS DE LA ISLA  
DE GRAN CANARIA**

**Rayco GUEDES ALONSO, Zoraida  
SOSA FERRERA y José Juan  
SANTANA RODRÍGUEZ**

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II Workshop "Estudio, aprovechamiento y gestión del agua en terrenos e islas volcánicas"  
Las Palmas de Gran Canaria, 2015

II Workshop "Estudio, aprovechamiento y gestión del agua en terrenos e islas volcánicas"

Las Palmas de Gran Canaria (España), enero de 2015



## Optimization of molecularly imprinted solid phase extraction (MISPE) coupled with UHPLC–FD, for the determination of estrogens in wastewaters

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### INTRODUCTION:



Female hormones, named estrogens, are considered as endocrine disrupting compounds (EDCs) and they are an important group of contaminants, among emerging pollutants which have attracted the attention of the international community due to their capacity of altering the natural hormonal equilibrium, producing harmful effects in organisms. Some authors have linked the concentrations of estrogens in environmental waters with changes observed in aquatic biota as for example changes in reproduction of fish [1]. The levels of EDCs in the environment are usually in the range of  $\text{ng L}^{-1}$  [2] so it is necessary the development of selective extraction methods.

Molecularly Imprinted Solid Phase Extraction (MISPE) is based in the use of a molecularly imprinted polymer as stationary phase of solid phase extraction which allows a selective extraction of a kind of compounds from a matrix.

In this study, a molecularly imprinted solid phase extraction (MISPE) coupled to ultra-high performance liquid chromatography with fluorescence detection has been optimized to determine four estrogens (estriol,  $17\beta$ -estradiol,  $17\alpha$ -ethynylestradiol and  $17\beta$ -estradiol-3-methyl ether) in wastewater samples.

### MATERIALS AND METHODS:

#### Chromatographic separation:

Mobile phase:

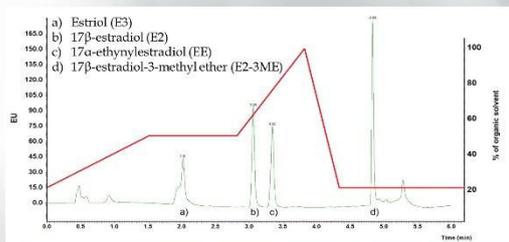
- A: Water + 0.1%  $\text{NH}_3$   
B: Acetonitrile

Chromatographic column:

- ACQUITY BEH C18 column  
(50 mm  $\times$  2.1 mm, 1.7  $\mu\text{m}$ )

#### SPE cartridges:

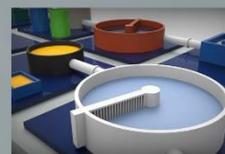
- Molecularly imprinted cartridges:  
- Affinimip® SPE Estrogens  
3 mL, 100 mg. of sorbent



Chromatogram of the compounds under study and the gradient used

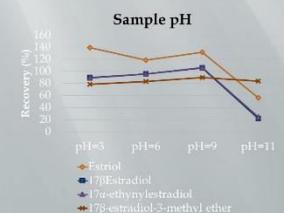
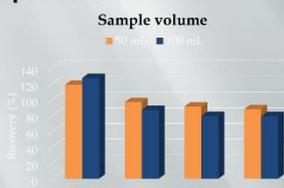
#### Sample collection:

Influent and effluent of a WWTP and effluent of a veterinary hospital in Gran Canaria (Spain)



### RESULTS:

#### Optimized variables:

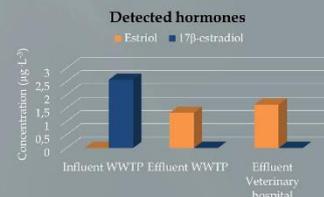


#### MISPE procedure:



#### Application to real samples:

	Recoveries (%)		
	Influent WWTP	Effluent WWTP	Effluent veterinary hospital
Estriol	40.0 $\pm$ 1.8	94.3 $\pm$ 4.1	87.0 $\pm$ 5.0
$17\beta$ -estradiol	80.7 $\pm$ 0.6	62.4 $\pm$ 9.7	105.8 $\pm$ 5.4
$17\alpha$ -ethynylestradiol	75.8 $\pm$ 2.5	60.0 $\pm$ 7.1	90.8 $\pm$ 3.3
$17\beta$ -estradiol 3 methyl ether	86.1 $\pm$ 4.3	66.0 $\pm$ 1.7	98.8 $\pm$ 3.5



### CONCLUSIONS

In accordance with the obtained results, the MISPE-UHPLC-FL procedure is easy, cheap, selective and sensitive, with low detection limits and good recoveries. The application in real sewage samples was satisfactory.

### REFERENCES

- [1] J.R. Colman, D. Baldwin, L.L. Johnson, N.L. Scholz, *Aquat. Toxicol.* 91 (2009) 346–354.  
[2] R. Guedes-Alonso, Z. Sosa-Ferrera, J.J. Santana-Rodríguez, *J. Anal. Methods Chem.* 2013 (2013) e210653.

SETAC Europe 25th Annual Meeting

Barcelona (España), mayo de 2015



## OPTIMIZATION OF MICROWAVE ASSISTED EXTRACTION COMBINED WITH UHPLC WITH FLUORESCENCE DETECTION FOR THE DETERMINATION OF ESTROGENS IN SLUDGE SAMPLES

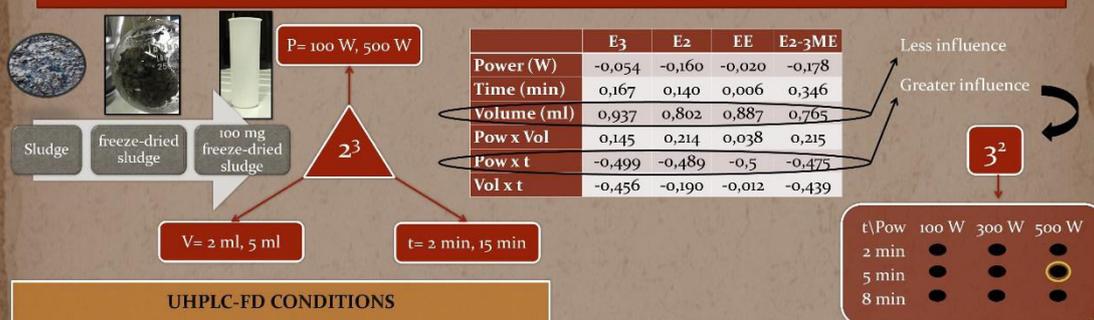


S. Santana-Viera, R. Guedes-Alonso, C. Afonso-Olivares, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J.J. Santana-Rodríguez.  
Departamento de Química, Universidad de Las Palmas de Gran Canaria, 35017, Las Palmas de Gran Canaria, Spain, e-mail:  
santana.viera@ulpgc.es

### INTRODUCTION

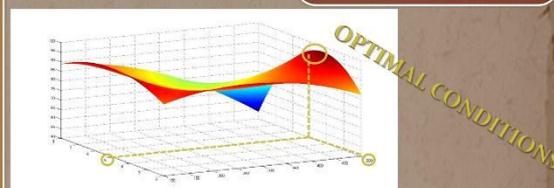
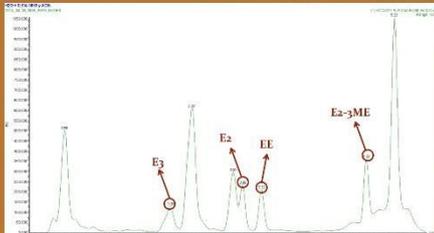
Emerging contaminants are a group of substances that has been detected at very low concentrations in the environment. Within this group of pollutants, this work is focused specifically on estrogens. Estrogens are a type of hormones that are proved to be endocrine disruptors. The conventional purifying treatments do not completely eliminate them and they are been adsorbed in the sludge [1]. New methodologies for extraction and preconcentration are required because these type of compounds are usually detected at trace level concentrations. Microwave assisted extraction (MAE) is a rapid technique which uses minimum volumes of solvents and it allows the extraction of analytes in complex matrices such as sludges [2]. In this work, it has been optimized a microwave assisted extraction combined with ultra-high performance liquid chromatography with fluorescence detection (UHPLC-FD) for the determination of a group of four estrogens in sludge samples from wastewater treatment plant.

### EXPERIMENTAL DESIGN



### UHPLC-FD CONDITIONS

- C<sub>18</sub> column
- Phase A: Mili-Q water with 0,1% NH<sub>3</sub>
- Phase B: ACN
- Gradient mode
- Excitation wavelength: 280 nm
- Emission wavelength: 310 nm



	RSD (%)	
	Interday	Intraday
E <sub>3</sub>	13,44	22,19
E <sub>2</sub>	17,85	18,16
EE	16,53	20,53
E <sub>2</sub> -3ME	18,05	11,04

### CONCLUSIONS

- A MAE-UHPLC-FD method for the determination of estrogens in sludge have been developed.
- All variables affecting the process such as time, power, volume of solvent and solvent, have been optimized.
- The Optimal Conditions were 10 ml of MeOH, 500 W and 5 min.
- Recoveries were around 50%.
- Interday RSD are below 20%, whereas Intraday RSD are between 20 – 30%.
- The method is suitable for determination of estrogens in sludge.

### REFERENCES

1. Guedes-Alonso, R., Montesdeoca-Esponda, S., Sosa-Ferrera, Z., Santana-Rodríguez, J.J., 2014. Trends Environ. Anal. Chem. 3-4, 14-27.
2. Vega-Morales, T., Sosa-Ferrera, Z., Santana-Rodríguez, J.J., 2013. Water. Air. Soil Pollut. 224, 1-15.

### ACKNOWLEDGMENT

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42<sup>nd</sup> International Symposium on High Performance Liquid Phase  
Separations and Related Techniques

Ginebra (Suiza), junio de 2015



## DETERMIANTION OF STEROID HORMONES IN WASTEWATERS BY USING FABRIC PHASE SORPTIVE EXTRACTION (FPSE) AND ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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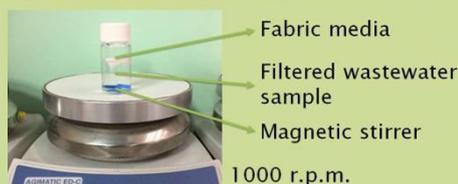
<sup>c</sup>International Forensic Research Institute, Department of Chemistry and Biochemistry, Florida International University, Miami, FL 33199, USA

### Introduction

Steroid hormones are considered as endocrine disrupting compounds (EDCs) due to their capability to affect the normal behavior of endocrine systems, even at very low concentrations, in the range of  $\text{ng} \cdot \text{L}^{-1}$ . To quantify these low concentrations, in this work we develop an extraction and preconcentration method by using fabric phase sorptive extraction (FPSE) followed by UHPLC-MS/MS. FPSE is a new technique which integrates the advantages of sol-gel derived microextraction sorbents and the rich surface chemistry of cellulose fabric, resulting in an efficient and solvent minimized sample preparation media [1]. Fabric phase sorptive extraction (FPSE) is appropriate for organic pollutants at trace concentration levels. The main advantages of this technique are the short extraction times and the minimum handling of sample. The proposed FPSE method has several advantages such as minimum use of organic solvents, short extraction times and high preconcentration factors. In this work, all the variables involved in the FPSE have been optimized and the developed method has been applied to sewage samples in order to determine the presence of 6 androgens and 4 progestogens

### Experimental

#### Fabric phase sorptive extraction



#### Chromatographic conditions

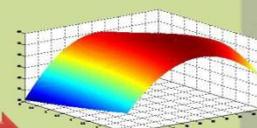
- Acquity UPLC system coupled to Triple Quadrupole Detector
- Column: Acquity BEH  $\text{C}_{18}$  (50x2.1 mm, 1.7  $\mu\text{m}$ )
- Mobile phase: Water+0.1%  $\text{NH}_3$  and Methanol
- Flow rate: 0.3  $\text{mL} \cdot \text{min}^{-1}$



### Results

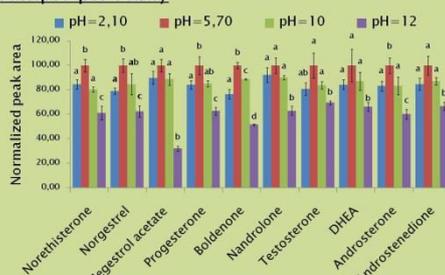
#### 2<sup>3</sup> experimental design

- Extraction time
  - Ionic strength
  - Desorption time
- Highest correlation



Optimum conditions:  
 •Extraction time: 20 min.  
 •Desorption time: 3 min  
 •Ionic strength: 0% NaCl

#### Sample pH study



Values with the same letter are not statistically different at 5% significance level according to the Dunnett T3 nonparametric test.

Compound	LOD <sup>1</sup> (ng · L <sup>-1</sup> )	Recovery <sup>2</sup> (10 ng · mL <sup>-1</sup> ) (%)	RSD <sup>2,3</sup> (%)	Concentration (ng · L <sup>-1</sup> ) in WWTP effluent
Norethisterone	33.5	49.2	9.2	< LOQ
Norgestrel	1.7	85.9	14.9	Not detected
Megestrol Ac.	23.4	61.7	6.0	< LOQ
Progesterone	6.9	72.3	0.6	< LOQ
Boldenone	46.9	70.8	14.6	< LOQ
Nandrolone	50.7	36.1	4.7	Not detected
Testosterone	2.2	33.2	5.6	55.6
DHEA	264	39.7	4.2	Not detected
Androsterone	63.6	92.0	0.2	Not detected
Androstenedione	19.4	40.4	4.1	< LOQ

<sup>1</sup>Limit of detection    <sup>2</sup>n = 6    <sup>3</sup>Relative standard deviation

### Conclusions

In accordance with the obtained results, the optimized FPSE procedure is easy, cheap, selective and sensitive, with low detection limits and comparable recoveries. The method was applied to wastewater samples.

### Aknowndgements

Rayco Guedes-Alonso would thank the University of Las Palmas de Gran Canaria for his PhD student grant

### References

- [1] R. Kumar, Gaurav, Heena, A.K. Malik, A. Kabir, K.G. Furton. J. Chromatogr A, 1359 (2014) 16.



42<sup>nd</sup> International Symposium on High Performance Liquid Phase Separations and Related Techniques

Ginebra (Suiza), junio de 2015



# ANALYSIS OF HORMONES IN SLUDGE SAMPLES USING MICROWAVE-ASSISTED EXTRACTION AND ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

R. Guedes-Alonso, S. Santana-Viera, C. Afonso-Olivares, S. Montesdeoca-Esponda, Z. Sosa-Ferrera, J.J. Santana-Rodríguez.

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## Introduction

Steroid hormones are natural and synthetic compounds which are considered as endocrine disruptor compounds due to their characteristics and possible toxic effects over aquatic biota [1]. Wastewater treatment plants (WWTPs) are the major sources of these compounds into the environment and because of their non-polar nature, they can be adsorbed on solid matrices. Nowadays, studies about determination of steroid hormones in solid samples are scarce in comparison with studies in liquid samples. However, as happen in liquid samples, concentrations of steroid hormones in solids are very low and it is necessary the development of methodologies of extraction and preconcentration which permits the determination of this kind of micropollutants at measurable concentrations [2]. Microwave-assisted extraction (MAE) is an easy technique which allows the extraction of analytes in complex matrices using small solvent volumes and short extraction times [3].

## Instrumental



Anton Paar Multiwave Microwave



Waters ACQUITY UHPLC-MS/MS

## Chromatographic and detection conditions

Separation in gradient mode

Column: ACQUITY BEH C18 (50x2.1 mm, 1,7µm)

Detection mode:

- ESI – for oestrogens
- ESI + for androgens, progesterones, glucocorticoids

Time (min)	%A	
	Water + 0.1 NH <sub>4</sub>	Methanol
0.00	80	20
2.75	25	75
3.75	0	100
6.00	80	20

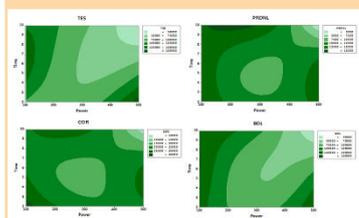
## Experimental

## Results

### MAE Optimization

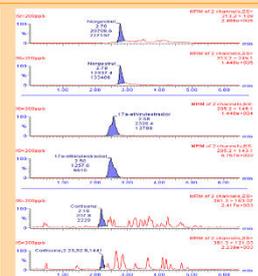
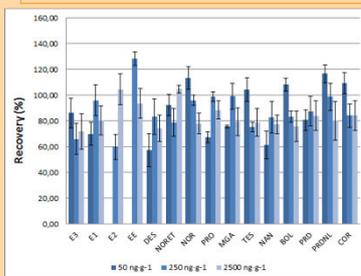
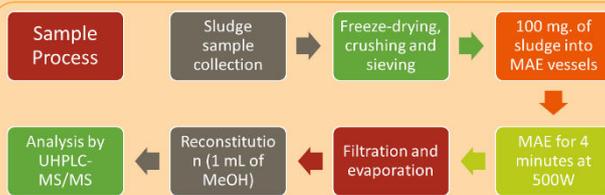
Experimental design

Power: 100-500W  
Extraction time: 2-10 min  
Extractant volume: 5-10 mL



Selective and sensitive method

LODs: 1.11 to 7.90 ng·g<sup>-1</sup>  
RSDs < 21%



## Conclusions

A MAE-UHPLC-MS/MS method for the determination of sex hormones and corticosteroids in sludge samples is presented for the first time. The method has appropriate detection limits, shows good selectivity and reproducibility and it has been satisfactorily applied to real samples.

Satisfactory applied to real samples

Norgestrel 430-1350 ng·g<sup>-1</sup>  
17α-ethynylestradiol 31.5-1440 ng·g<sup>-1</sup>  
Cortisone 17.3 ng·g<sup>-1</sup>

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## STUDY OF THE PRESENCE OF STEROID STEROID HORMONES RESIDUES IN FISH TISSUES FROM GRAN CANARIA (SPAIN)

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### INTRODUCTION

Steroid hormones are a worrisome group of compounds because of their toxic effects over aquatic biota. These compounds are considered as endocrine disrupting compounds because they can produce important changes not only in sexual systems of aquatic organisms but also in other biological systems [1]. These changes could be produced at very low concentrations, sometimes in the range of ng·L<sup>-1</sup>. This work presents an analytical method based in microwave-assisted extraction (MAE) coupled to ultra-high performance liquid chromatography and mass spectrometry detection (UHPLC-MS/MS) which constitutes a powerful procedure for the analysis of steroid hormones in solid and biological samples. The method was applied to three different types of tissue samples of two fishes of the coast of the Canary Islands (*Boops boops* and *Sphaeroides marmoratus*) exposed to the marine outfall of the Las Palmas de Gran Canaria city wastewater treatment plant.

### MATERIAL & METHODS



Anton Paar Multiwave Microwave



Waters ACQUITY UHPLC-MS/MS

Fish collection, separation of tissues, freeze-drying, and crushing

100 mg. of fish tissue into MAE vessels

MAE for 7 minutes at 450W

Filtration and remove proteins by SPE (Phenomenex Phree cartridges)

Analysis by UHPLC-MS/MS

### RESULTS

#### Fish species analyzed



*Boops boops*



*Sphaeroides marmoratus*

#### Sample location



Marine outfall of Barranco Seco WWTP



#### Detected concentrations of hormones (µg·g<sup>-1</sup>)

Compound	<i>Boops boops</i>			<i>Sphaeroides marmoratus</i>		
	Muscle	Viscera	Skin	Muscle	Viscera	Skin
Norgestrel	< LOQ	2.71	n.d.	< LOQ	0.50	n.d.
Testosterona	< LOQ	n.d.	n.d.	< LOQ	n.d.	n.d.
Megestrol acetate	< LOQ	n.d.	< LOQ	< LOQ	n.d.	< LOQ
Prednisolone	n.d.	n.d.	0.09	n.d.	n.d.	n.d.
Boldenone	< LOQ	< LOQ	n.d.	< LOQ	< LOQ	n.d.
Norethisterone	< LOQ	n.d.	n.d.	< LOQ	n.d.	n.d.
Nandrolone	< LOQ	0.52	n.d.	n.d.	0.07	n.d.
Progesterone	< LOQ	3.22	1.77	0.56	3.26	3.95

### CONCLUSIONS

The method developed (MAE-SPE-UHPLC-MS/MS) enables easy and quick determination of steroid hormones from fish tissue in less than 30 minutes. After application of the method to different fish tissues, concentrations detected in viscera and skin were higher than the concentrations in muscle tissue.

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