UNIVERSITAT JAUME·I

POTENCIAL DE LA CROMATOGRAFÍA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE MASAS EN TÁNDEM PARA LA DETECCIÓN, CUANTIFICACIÓN Y CONFIRMACIÓN DE FÁRMACOS EN AGUAS

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Tesis Doctoral

EMMA GRACIA LOR

2013

Los Dres. **Félix Hernández Hernández** y **Juan Vicente Sancho Llopis**, Catedráticos de Química Analítica de la Universitat Jaume I de Castellón,

Certifican: que la Tesis Doctoral "Potencial de la cromatografía líquida acoplada a espectrometría de masas en tándem para la detección, cuantificación y confirmación de fármacos en aguas" ha sido desarrollada bajo su dirección, en el Instituto Universitario de Plaguicidas y Aguas, Departament de Química Física i Analítica de la Universitat Jaume I de Castellón, por **Emma Gracia Lor**.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 25 de marzo de 2013.

Fdo. Dr. Félix Hernández Hernández Fdo. Dr. Juan Vicente Sancho Llopis

Este trabajo se ha realizado mediante la concesión de una beca predoctoral de la Universitat Jaume I para la formación de personal investigador, desde 1 de julio de 2008.

Emma Gracia Lor ha sido beneficiaria de una beca concedida por la Universitat Jaume I para la realización de una estancia en el *Istituto di Ricerche Farmacologiche "Mario Negri"* de Milán, desde el 15 de marzo al 20 de junio de 2012. El trabajo realizado llevó por título "Investigación de fármacos y metabolitos en aguas mediante técnicas de análisis basadas en la espectrometría de masas" y se llevó a cabo bajo la supervisión de la Dra. Sara Castiglioni. La estancia en este centro de investigación permitió a la doctoranda profundizar en los aspectos analíticos relacionados con la presencia de este tipo de compuestos en el medio ambiente así como estudiar la capacidad de los sistemas de depuración para la eliminación de fármacos y otros contaminantes relacionados.

Esta tesis ha sido realizada, y consecuentemente será defendida, con el propósito de obtener el título de Doctorado Internacional.

Previamente a la defensa de la Tesis Doctoral, este trabajo ha sido evaluado por dos censores extranjeros independientes, Dra. Barbara Kasprzyk-Hordern *(Department of Chemistry, University of Bath, UK)* y Dr. Ettore Zuccato *(Dipartimento di Ambiente e Salute, Istituto di Ricerche Farmacologiche Mario Negri*, Milán, Italia).

A mis padres y hermano

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Resumen

Los fármacos son probablemente los contaminantes emergentes que en los últimos años han suscitado un mayor interés debido a que su presencia en el medio ambiente puede afectar a la calidad del agua y a los organismos vivos. Tras su consumo humano y/o veterinario, los fármacos pueden excretarse sin sufrir ninguna modificación o bien en forma de metabolitos, llegando a alcanzar concentraciones importantes en las aguas residuales urbanas. Los tratamientos aplicados en la mayoría de las estaciones depuradoras de aguas residuales no son suficientes para eliminarlos completamente, con lo que finalmente llegan al medio acuático. Se requiere pues metodología analítica que permita determinar de modo fiable los fármacos y sus metabolitos en el medio acuático para poder evaluar sus posibles efectos negativos sobre el medio ambiente.

En esta Tesis se ha estudiado el potencial analítico y las aplicaciones del acoplamiento instrumental cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS) con analizadores de triple cuadrupolo (QqQ) y con analizador híbrido cuadrupolo-tiempo de vuelo (QTOF) en la determinación de fármacos y de sus metabolitos/productos de transformación aguas de distinta naturaleza.

El trabajo realizado se ha dividido en dos partes. En primer lugar, se ha estudiado el potencial del acoplamiento UHPLC-MS/MS para la determinación cuantitativa de fármacos. Se han desarrollado tres métodos multirresiduales basados en una etapa de tratamiento de muestra mediante SPE y posteriormente determinación por UHPLC-MS/MS con triple cuadrupolo. En todos ellos se han determinado simultáneamente compuestos pertenecientes a distintas familias de fármacos, con propiedades físico-químicas muy diversas, en una única inyección; es decir, se determinan simultáneamente los compuestos que se ionizan en modo positivo y en modo negativo.

En el primer método se seleccionaron los 20 fármacos más consumidos en España. Se analizaron compuestos como ibuprofeno, diclofenaco, atorvastatina, omeprazol, alprazolam, entre otros. Posteriormente, esta lista de compuestos se amplió

notablemente mediante la incorporación de aproximadamente 30 antibióticos, dando como resultado el método presentado en el segundo trabajo. La elección de estos compuestos se basó fundamentalmente en los posibles efectos negativos que pueden ocasionar en los organismos vivos y en el medio acuático. Ambos métodos fueron optimizados y validados tanto en agua superficial como en aguas residuales urbanas. Ambas metodologías se aplicaron al análisis de muestras de influentes y de efluentes con el fin de estudiar la presencia de los fármacos seleccionados, así como su posible eliminación en estaciones depuradoras de aguas urbanas situadas en la provincia de Castellón en las que se utilizan tratamientos convencionales. Destacó la presencia de los analgésicos/antiinflamatorios y los fármacos utilizados para el tratamiento del colesterol y los antibióticos pertenecientes al grupo de las quinolonas. En cuanto al comportamiento de los fármacos durante el proceso de tratamiento, se pudo diferenciar entre aquellos compuestos que se eliminaron completamente, un grupo mucho más numeroso formado por analitos cuya eliminación fue incompleta y un último grupo constituido por aquellos compuestos que únicamente se detectaron en las muestras de efluente.

La primera parte de esta Tesis finaliza con un tercer método para el análisis de fármacos seleccionados y productos de cuidado personal. El interés por este último tipo de compuestos ha aumentado en los últimos años, motivo por el cual se decidió incorporarlos a la lista de contaminantes objeto de estudio. En todos los trabajos realizados, pero especialmente en éste, se puso especial interés en la evaluación del efecto matriz en diferentes tipos de agua para la correcta cuantificación de los analitos. Se realizó un estudio en profundidad de la eficacia del proceso de extracción por SPE y del efecto matriz en 10 muestras de agua diferentes. El método se validó así mismo en varios tipos de aguas superficiales y de efluentes con el objetivo de afrontar diferentes composiciones de matriz y situaciones reales que pueden aparecer cuando se analizan muestras de agua. Finalmente, en un estudio colaborativo con la Universidad de Antioquia, Medellín, se analizaron 73 muestras (aguas superficiales y efluente) recogidas en España y en Colombia. En este último caso, las muestras procedían de dos embalses utilizados para el abastecimiento de agua potable y tan sólo se detectaron productos de cuidado personal. La presencia de estos compuestos también resultó

destacable en las aguas superficiales recogidas en la Comunidad Valenciana. En ellas también se hallaron también casi todos los fármacos seleccionados. En el caso de las aguas de efluente, aproximadamente el 65% de los compuestos se detectaron en todas las muestras. Cabe destacar los elevados niveles de concentración de los fármacos analizados, a diferencia de los compuestos de cuidado personal cuyos niveles de concentración resultaron más bien bajos.

La segunda parte de la Tesis se centra en la investigación de metabolitos/productos de transformación de fármacos en aguas. Esta línea de investigación se inició porque algunos fármacos analizados en los primeros trabajos no se habían detectado en ninguna de las muestras. Llamó especialmente la atención en el caso de fármacos que se habían seleccionado por su elevado consumo. Por ello, se decidió investigar la presencia de sus metabolitos y/o productos de transformación en las aguas. Además, la información que existe actualmente acerca de la presencia de los metabolitos de fármacos en el medio ambiente y de sus posibles efectos es escasa, a pesar de que estos compuestos deberían tenerse en consideración ya que una exposición prolongada podría tener efectos indeseados similares a los de los fármacos.

En primer lugar, se estudió el potencial del acoplamiento UHPLC-QTOF MS en la investigación de aproximadamente 160 metabolitos/TPs, reportados en la bibliografía, en muestras de efluente en las que previamente se habían detectado diversos fármacos (análisis retrospectivo). Los análisis se realizaron utilizando el modo de trabajo MS^E, que implica la adquisición simultánea de dos funciones a diferentes energías de colisión (alta y baja energía) y permite adquirir simultáneamente información sobre el ión molecular y sus iones fragmento en una única inyección. La evaluación retrospectiva permitió la detección e identificación de cuatro metabolitos, en concreto, N-desmetil claritromicina y 14-hidroxi-claritomicina, ambos metabolitos de la claritromicina, ácido fenofíbrico y ácido carboxílico de clopidogrel, metabolitos del fenofibrato y de clopidogrel respectivamente.

Por otro lado, la elevada resolución y exactitud de masa del QTOF permitió resolver una situación problemática observada en análisis previos realizados por LC-

MS/MS (QqQ), que habían dado lugar a falsos positivos de uno de los metabolitos del fármaco dipirona (4-AA). El uso de la alta resolución nos ayudó a descubrir la presencia de otros dos metabolitos de la dipirona que compartían las mismas transiciones, complicando de este modo la correcta identificación de los compuestos. Además, este estudio sirvió para resaltar la importancia que tiene la separación cromatográfica en situaciones problemáticas en las que los metabolitos de un compuesto pueden compartir las mismas transiciones. En el caso de la dipirona, los metabolitos descubiertos por QTOF, que compartían las mismas transiciones que el previamente analizado 4-AA, fueron el 4-formilaminoantipirina (4-FAA) y 4-acetamidoantipirina (4-AAA). Aunque con una estructura química y fórmula molecular diferentes, ambos sufrieron fragmentación en la fuente electrospray, dando lugar al 4-AA, que fue el compuesto reportado en los análisis realizados mediante UHPLC-MS/MS.

Finalmente, se desarrolló un método basado en UHPLC-MS/MS para la determinación cuantitativa de varios metabolitos identificados mediante QTOF. Además, se seleccionaron otros metabolitos reportados en las aguas según la bibliografía, y se incluyeron también sus fármacos de partida. El método se validó en agua superficial y de efluente. En este último caso, gracias a la elevada sensibilidad del método se pudo corregir el efecto matriz mediante una simple dilución de las muestras (x4). De los 21 compuestos analizados, tan sólo tres (ácido clofíbrico, enalaprilato y omeprazol) no se hallaron en ninguna de las muestras. Esta información demuestra la importancia que tiene la inclusión de los metabolitos/TPs en los métodos multirresiduales, ya que se detectan con elevada frecuencia en las aguas y, en ocasiones, a concentraciones superiores a las de los propios fármacos.

Summary

Pharmaceuticals are among the emerging contaminants that have caused a greater interest in the last years because of their presence in the environment can affect the water quality and living organisms. After their human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form as the parent compound and/or as metabolites, reaching high concentrations in urban wastewaters. Treatment processes applied in most wastewater treatment plants are not efficient enough to remove pharmaceuticals completely, which can eventually reach the aquatic environment. Therefore, analytical methodology is required to determine pharmaceuticals and their metabolites in the aquatic ecosystem in a reliable way to evaluate their potential negative effects on the environment.

In this Thesis the analytical potential and applications of liquid chromatography and tandem mass spectrometry (LC-MS/MS) with triple quadrupole (QqQ) and hybrid quadrupole-time of flight (QTOF) analyzers in the determination of pharmaceuticals and their metabolites/transformation products in different types of waters has been studied.

The work done has been divided in two parts. Firstly, the potential of the coupling UHPLC-MS/MS has been explored for the quantitative determination of pharmaceuticals. Three multi-residue methods based on a pre-treatment step by solid-phase extraction followed by UHPLC-MS/MS determination with triple quadrupole have been developed. In all of them, compounds belonging to different pharmaceutical groups, with very diverse physico-chemical characteristics have been determined in a single injection, i.e, positive and negatively ionized compounds are simultaneously determined in a single analysis.

In the first method, the 20 most consumed pharmaceuticals in Spain were selected. Compounds such as ibuprofen, diclofenac, atorvastatin, omeprazol, alprazolam, among others, were analyzed. Later, this list of compounds was notably enlarged by the addition of around 30 antibiotics, resulting in the method presented in the second work. These compounds were mainly selected due to their potential negative

effects on living organisms and on the aquatic environment. Both methods were optimized and validated in surface waters and in urban wastewaters. Subsequently, both methodologies were applied to the analysis of influent and effluent wastewaters in order to study the presence of selected pharmaceuticals, as well as their possible removal, in urban wastewater treatment plants located in the Castellon province where conventional treatments are applied. Analgesics/anti-inflammatories and compounds used to lower cholesterol levels and quinolone antibiotics were the most frequently detected pharmaceuticals. Regarding their behaviour during the treatment process, we could distinguish among those compounds completely removed, a numerous group formed by the analytes that were partially removed, and a last group including compounds that were only detected in the effluent wastewater samples.

The first part of Thesis ends with a third method developed for the analysis of selected pharmaceuticals and personal care products. The interest regarding the last family of compounds has increased in the last years, therefore they were included in the list of contaminants under study. In all the works carried out, but mainly in this one, special interest was paid on the evaluation of matrix effects in different types of water for the correct quantification of the analytes. A detailed study of the extraction process efficiency and matrix effect was carried out in 10 different water samples. The method was validated in several types of surface waters and effluents in order to evaluate different matrix compositions and real situations that can appear when real-world water samples are analyzed. Finally, in a collaborative study with the Antioquia University, (Medellin) 73 samples (surface and effluent) collected in Spain and Colombia were analyzed. In the last case, the samples were collected from two reservoirs used for drinking water supply and only personal care products were detected. The presence of these compounds was also remarkable in the surface water samples collected in the Valencian Community (Spain). In these samples, almost all the target pharmaceuticals included in the method were found. In the case of effluent wastewater samples, approximately 65% of the compounds were detected in all the samples. It is important to remark the high concentration levels found for some pharmaceuticals, in contrast to the personal care products which concentrations were rather low.

The second part of the Thesis is focused on the investigation of pharmaceutical metabolites/transformation products in waters. This research was made because some of the pharmaceuticals selected in the first works were never detected in any of the samples analyzed. It was especially surprising in the case of pharmaceuticals highly consumed. So, we decided to study the presence of their metabolites and/or transformation products in water. Moreover, the information currently available regarding the presence of pharmaceutical metabolites in the environment and their possible effects is scarce, despite these compounds are also of concern as a long exposition may have undesirable effects similar to those of the parent pharmaceuticals.

Firstly, the potential of UHPLC-QTOF MS has been evaluated in the investigation of around 160 metabolites/TPs, reported in the bibliography, in effluent wastewater samples where several pharmaceuticals had been previously detected (retrospective analysis). Analyses were made in MS^E mode, which involves the simultaneous acquisition of two functions at different collision energies (low and high). This allows acquiring simultaneously information about the molecular ion and the fragment ions in a single injection. The retrospective evaluation allowed the detection and identification of four metabolites, such as N-desmethyl clarithromycin and 14-hydroxy-clarithromycin, both metabolites of clarithromycin, fenofibric acid and clopidogrel carboxylic acid, metabolites of fenofibrate and clopidogrel, respectively.

On the other hand, the high resolution and exact mass measurements of the QTOF allowed to solve a problematic situation observed in previous analysis by LC-MS/MS (QqQ), which had led to false positives of one of the metabolites (4-AA) of the pharmaceutical dipyrone. The use of high resolution helped us to discover the presence of two other dipyrone metabolites that shared the same transitions, complicating the right identification of the compounds. Moreover, this study was useful to highlight the importance of the chromatographic separation in problematic situations where the metabolites of a compound can share the same transitions. In the case of dipyrone, the metabolites discovered by QTOF, that showed the same transitions than the previously analyzed 4-AA, were 4-formilaminoantipyrine (4-FAA) and 4-acetamidoantipyrine (4-AAA). Although they have different chemical structure and molecular formula, both

suffered in-source fragmentation in the electrospray source, producing 4-AA, the compound that had been reported in the analysis carried out by UHPLC-MS/MS.

Finally, a method based on UHPLC-MS/MS was developed for the quantitative determination of several metabolites identified by QTOF. Moreover, other metabolites detected in waters according to the consulted bibliography, as well as their parent compounds were also included. The method was validated in surface and effluent wastewater. In this last case, the high sensitivity of the method allowed to correct matrix effects by a simply 4-fold dilution of the samples. Only 3 out of 21 compounds analyzed (clofibric acid, enalaprilate and omeprazole) were not found in any of the samples. Our data showed the importance of including pharmaceutical metabolites/TPs in the multi-residue methods, as they are frequently detected in waters and, sometimes, at concentrations higher than their parent compounds.

ÍNDICE GENERAL

Índice de acrónimos

APCI	Interfase de Ionización Química a Presión Atmosférica
ATC	Sistema de clasificación Anatómica, Terapéutica y Química
CID	Disociación Inducida por Colisión
DDD	Dosis Diarias Definidas
DEET	N,N-Dietil-meta-toluamida
EDAR	Estación Depuradora de Aguas Residuales
EDTA	Ácido Etilendiaminotetraacético
EMEA	Agencia Europea del Medicamento
EPA	Agencia de Protección Ambiental de los Estados Unidos
ESAC	Vigilancia Europea del Consumo de Antimicrobianos
ESI	Interfase Electrospray
FDA	Agencia de Alimentos y Medicamentos de EEUU
FEDESA	Federación Europea de Salud Animal
GC	Cromatografía de Gases
GC-MS	Cromatografía de gases acoplada a la espectrometría de masas
HPLC	Cromatografía Líquida de Alta Resolución
IP	Punto de Identificación
IUPAC	Unión Internacional de Química Pura y Aplicada
LC	Cromatografía Líquida
LC-MS	Cromatografía Líquida acoplada a la espectrometría de masas
LC-MS/MS	Cromatografía de líquidos acoplada a la espectrometría de masas en tándem
LOD	Límite de Detección
LOQ	Límite de Cuantificación
LPME	Microextracción en fase líquida
MIP	Polímero Molecularmente Impreso
MRL	Límite Máximo de Residuos
MS	Espectrometría de Masas

MS/MS	Espectrometría de Masas en tándem
m/z	Relación masa/carga
OMS	Organización Mundial de la Salud
PCP	Producto de Cuidado Personal
ppb	Partes por billón
PPCP	Fármaco y Producto de Cuidado Personal
QC	Control de Calidad
QqQ	Analizador de Triple Cuadrupolo
QTOF	Analizador híbrido Cuadrupolo-Tiempo de Vuelo
SBSE	Extracción por adsorción con Barras Magnéticas Agitadoras
SIM	Monitorización del ion seleccionado
S/N	Relación señal/ruido
SPE	Extracción en fase sólida
SPME	Microextracción en fase sólida
SRM	Monitorización de la transición seleccionada
TIC	Cromatograma de Iones Totales
TOF	Analizador de Tiempo de Vuelo
TP	Producto de Transformación
UHPLC	Cromatografía líquida de ultra resolución
XIC	Cromatograma del Ión Extraído

Objetivos, metodología y plan de trabajo

Objetivos

El **principal objetivo** de la presente Tesis Doctoral es explorar las capacidades analíticas de la cromatografía líquida acoplada a la espectrometría de masas en tándem (LC-MS/MS) con analizadores de triple cuadrupolo (QqQ) y cuadrupolo-tiempo de vuelo (QTOF) para la determinación de fármacos y sus metabolitos/productos de transformación en aguas.

La Tesis se divide en dos partes bien diferenciadas:

- La primera parte se centra en la determinación cuantitativa de fármacos en aguas mediante UHPLC-MS/MS con analizador de triple cuadrupolo. El objetivo principal es el desarrollo, validación y aplicación de nuevos métodos analíticos para la determinación de numerosos fármacos de muy diversas familias químicas, en aguas de distinta naturaleza.

- La segunda parte se centra en la investigación de metabolitos y/o productos de transformación de fármacos en aguas de distintos tipos, tanto desde un enfoque cualitativo (utilizando un analizador QTOF) como cuantitativo (QqQ).

Con el fin de alcanzar este objetivo principal se han establecido los siguientes **objetivos específicos**:

1. Desarrollar metodología analítica avanzada para la determinación multirresidual de fármacos -seleccionados por su mayor consumo y/o impacto ambiental- en aguas, basada en una etapa de extracción en fase sólida (SPE) seguida de determinación mediante UHPLC-MS/MS con analizador de triple cuadrupolo.

2. Estudiar las condiciones de SPE que permitan la extracción simultánea y eficiente de todos analitos seleccionados.

3. Aplicar la cromatografía líquida de ultra presión (UHPLC), tanto en métodos MS/MS como QTOF MS, para realizar análisis rápidos, con buena resolución cromatográfica, y que permitan la determinación simultánea de los compuestos ionizados en modo positivo y negativo en una sola inyección.

4. Evaluar el efecto matriz en métodos cuantitativos basados en LC-MS/MS para diversos tipos de agua y establecer la corrección que resulte más adecuada en los métodos multirresiduales desarrollados, prestando especial atención al uso de patrones internos marcados isotópicamente.

5. Aplicar la metodología analítica desarrollada a muestras acuosas, principalmente aguas superficiales y aguas residuales urbanas procedentes de distintas EDAR situadas en la Comunidad Valenciana. Con la información obtenida se persigue obtener una visión realista sobre la calidad del agua de esta zona, al estar centrados los análisis sobre los fármacos más consumidos. Asimismo, se pretende evaluar la eficacia de los tratamientos convencionales aplicados en las EDAR para la eliminación de fármacos.

6. Desarrollar metodología analítica cualitativa basada en UHPLC-QTOF MS para la identificación *post-target* de metabolitos/productos de transformación de fármacos en muestras de agua.

7. Utilizar la información obtenida mediante UHPLC-QTOF MS para establecer los metabolitos/productos de transformación de interés y desarrollar posteriormente un método analítico que permita su determinación cuantitativa mediante LC-MS/MS con analizador QqQ.

Metodología y plan de trabajo

La metodología de trabajo seguida para el desarrollo de métodos analíticos cuantitativos ha sido la siguiente:

1. Selección de los fármacos más relevantes tanto desde el punto de vista del consumo humano como del riesgo medioambiental.

2. Optimización de las condiciones de MS y MS/MS mediante la infusión individual de los patrones analíticos.

   - Adquisición de los espectros MS en modo barrido (*scan*) estableciendo el modo de ionización y el voltaje de cono para el ión precursor.

   - Intentar favorecer la formación de la molécula protonada (modo de ionización positivo) mediante la adición de aditivos compatibles con el sistema (ácido fórmico o acetato amónico) cuando sea necesario.

   - Aislamiento del ión precursor y optimización de la energía de colisión para la obtención de los iones producto característicos.

   - Selección de los iones producto teniendo en cuenta la sensibilidad (abundancia del ión) y la selectividad (especificidad de la transición), evitando en la medida de lo posible las transiciones derivadas de pérdidas genéricas como, por ejemplo, agua, dióxido de carbono, ácido fórmico, cloro, etc.

   - Adquisición de, al menos, dos transiciones MS/MS por compuesto para facilitar la correcta identificación de los compuestos detectados en las muestras.

3. Optimización de la separación cromatográfica mediante la inyección de patrones en disolución. Elección de la fase móvil y del gradiente para obtener picos cromatográficos y tiempos de retención adecuados.

4. Aplicación y estudio de la eficacia de SPE para la extracción y preconcentración de las muestras, mediante el análisis de muestras blanco fortificadas antes y después de la extracción y de patrones de la misma concentración para evaluar las posibles pérdidas durante el proceso de extracción.

5. Estudio del efecto matriz en los métodos LC-MS/MS desarrollados mediante el análisis de muestras fortificadas de diversos tipos y procedencias, así como de patrones en solvente. Estudio de posibles soluciones que no supongan un aumento del tiempo de análisis ni de la manipulación de la muestra y que resulten adecuadas para un método multirresidual. Importancia del uso de patrones internos marcados isotópicamente.

6. Validación del los métodos desarrollados y optimizados evaluando la linealidad, exactitud y precisión mediante ensayos de recuperación a varios niveles de concentración. Estimación del límite de detección y del límite de cuantificación del método.

7. Aplicación de la metodología analítica desarrollada al análisis de aguas de distinta naturaleza (influente, efluente y superficial).

8. Confirmación de la identidad de los compuestos detectados en las muestras mediante el cálculo de la relación iónica (intensidad de la señal para las transiciones seleccionadas) en muestras y patrones.

9. Discusión de los resultados obtenidos y establecimiento de conclusiones relativas a la presencia de fármacos en las aguas analizadas.

La metodología general seguida para el <u>análisis cualitativo</u> ha sido la siguiente:

1. Inyección de la muestra en UHPLC-QTOF y adquisición del espectro de masas *full scan* en modo MS^E a baja y a alta energía de colisión.

2. Extracción de la masa exacta de la molécula protonada (o desprotonada) de cada compuesto a partir del espectro a baja energía, originando un cromatograma de ión extraído (XIC) donde la presencia de un compuesto en la muestra conduce al correspondiente pico cromatográfico.

3. Cálculo del error de la masa experimental con respecto a la masa exacta teórica.

4. Identificación de los iones fragmento más relevantes en el espectro a alta energía (si el compuesto investigado está realmente presente en la muestra, el tiempo de retención de los picos cromatográficos obtenidos al realizar XICs a las masas exactas de los fragmentos debe ser el mismo que el de la molécula protonada y mostrar la misma forma de pico).

5. Cálculo del error de masa de los iones fragmento.

6. Deducción de sus estructuras mediante el software adecuado (MassFragment) o por predicción, teniendo en cuenta las diferencias estructurales con el fármaco de partida, y evaluación de la información mediante búsqueda bibliográfica.

7. Análisis de muestras de agua e investigación de metabolitos/TPs de fármacos en las mismas siguiendo la metodología desarrollada. Identificación tentativa sobre la base de la información aportada por QTOF MS.

8. En el caso de disponer de patrones comerciales, inyección y comparación de sus masas exactas, iones fragmento y tiempos de retención, con el fin de proceder a la identificación inequívoca del metabolito detectado.

El **plan de trabajo** seguido se indica a continuación:

1. Selección de los compuestos a estudiar en base a varios criterios: fármacos más consumidos en España con receta médica, según la información proporcionada por el Ministerio de Sanidad; compuestos seleccionados debido a sus efectos potencialmente negativos en los organismos vivos del medio acuático; fármacos detectados en agua superficial y residual en estudios publicados por otros autores.

2. Revisión bibliográfica sobre los métodos de análisis existentes para la determinación de fármacos mediante la técnica LC-MS/MS.

3. Desarrollo y optimización de dos métodos analíticos multirresiduales en aguas para la determinación simultánea de fármacos con diferentes características físico-químicas pertenecientes a distintas clases terapéuticas. Análisis cromatográfico rápido mediante el uso de la técnica UHPLC. Estudio del efecto de la matriz y corrección del mismo. Validación de los métodos desarrollados.

4. Aplicación de los métodos desarrollados a aguas superficiales recogidas en diferentes puntos de la Comunidad Valenciana y a muestras de aguas residuales (influente y efluente) procedentes de distintas EDAR situadas en la provincia de Castellón.

5. Desarrollo, optimización y validación de un método basado en UHPLC-MS/MS para la determinación simultánea de fármacos frecuentemente detectados y de productos de cuidado personal. Estudio detallado de la eficacia del proceso de extracción mediante SPE y del efecto de la matriz en distintos tipos de agua. Corrección de las pérdidas de extracción y de la exaltación/supresión de la señal.

6. Aplicación del método anterior al análisis de muestras recogidas en España y en Colombia en un estudio colaborativo con la Universidad de Antioquia.

7. Búsqueda de metabolitos de fármacos en aguas de efluente previamente analizadas por UHPLC-QTOF MS en modo MS^E, realizando un análisis retrospectivo de los datos adquiridos (masa exacta, espectro completo).

8. Utilización de la espectrometría de masas de alta resolución (QTOF MS) para la identificación de metabolitos que comparten los mismos fragmentos. Se incluyen en este apartado metabolitos del fármaco dipirona, como ejemplo ilustrativo.

9. Desarrollo y validación de un método UHPLC-MS/MS para la determinación cuantitativa de un número notable de metabolitos identificados por QTOF MS, así como de otros metabolitos detectados en las aguas según la literatura científica, junto con sus fármacos de procedencia.

10. Aplicación de la metodología analítica desarrollada al análisis cuantitativo de fármacos y metabolitos en aguas superficiales y residuales.

11. Elaboración de las principales conclusiones derivadas de las investigaciones realizadas en esta Tesis Doctoral.

Objectives, methodology and working plan

Objectives

The **main objective** of this Thesis is to investigate the analytical capabilities of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), using triple quadrupole (QqQ) and quadrupole-time of flight (QTOF) analysers, for the determination of pharmaceuticals and their metabolites/transformation products in water.

The Thesis is divided in two parts according to this objective:

- The first part is focused on the quantitative determination of pharmaceuticals in waters using UHPLC-MS/MS with triple quadrupole. The main objective is the development, validation and application of new analytical methods for the determination of numerous pharmaceuticals belonging to different chemical families, in various types of water samples.

- The second part is focused on the investigation of metabolites and/or transformation products of pharmaceuticals in different types of water. The study if focused from a qualitative (using a QTOF analyzer) and a quantitative (QqQ) point of view.

In order to reach this main objective, the following **specific objectives** have been established:

1. Development of advanced analytical methodology for multi-residue determination of pharmaceuticals, selected on the basis of their high consumption and/or environmental impact, in waters based on a solid phase extraction (SPE) step followed by determination by UHPLC-MS/MS with triple quadrupole.

2. Study the SPE conditions that allow the simultaneous and efficient extraction of the selected analytes.

3. Application of ultra-high performance liquid chromatography (UHPLC), in MS/MS methods and in QTOF MS, to achieve rapid analysis, with good chromatographic resolution, that allows the simultaneous determination of the compounds ionized under positive and negative mode in only one injection.

4. Evaluation of matrix effects in quantitative LC-MS/MS methods for different types of waters. Correction of matrix effects, paying special attention to the use of isotope-labelled internal standards.

5. Application of the developed analytical methodology to aqueous samples, mainly surface water and urban wastewater samples from different WWTPs located in the Valencian Community. A realistic overview of the water quality in this area is pursued from the obtained information, as analyses are focused on the most consumed pharmaceuticals. Furthermore, the efficiency of conventional treatments applied in the WWTPs to eliminate pharmaceuticals is also assessed.

6. Development of qualitative analytical methodology based on UHPLC-QTOF MS for the post-target detection and identification of metabolites/transformation products of pharmaceuticals in waters

7. Use the information obtained by UHPLC-QTOF MS to establish the metabolites/transformation products of interest and subsequent development of analytical methodology for their quantitative determination based on LC-MS/MS with QqQ analyzer.

Methodology and working plan

The methodology applied for quantitative methods development is the following:

1. Selection of the most relevant pharmaceuticals from the consumption and environmental impact point of views.

2. Optimization of MS and MS/MS conditions by infusion of individual analytical standards.

    - Acquisition of the MS spectra in scan mode establishing the ionization mode and the cone voltage for the ion precursor.

    - Improvement of the formation of the protonated molecule (positive ionization mode) by using additives compatibles with the system (formic acid or ammonium acetate) when necessary.

    - Isolation of the precursor ion and optimization of the collision energy to obtain the characteristics product ions.

    - Selection of the most appropriate product ions taking into account the sensitivity (ion abundance) and selectivity (specifity of the transition), trying to avoid those transitions derived from common losses as, for example, water, carbon dioxide, formic acid, chlorine, etc.

    - Acquisition of, at least, two MS/MS transitions per compound to facilitate the correct identification of the compounds detected in the samples.

3. Optimization of the chromatographic separation by injecting standard solutions. Selection of the mobile phase and the gradient in order to obtain suitable chromatographic peaks and retention times.

4. Study of the SPE process efficiency to extract and pre-concentrate the water samples, from the analysis of blank samples spiked before and after the SPE step and of standards of the same concentration in order to evaluate the potential losses during the SPE process.

5. Study of the matrix effect in the LC-MS/MS methods developed by analysing spiked samples of different types and origin, as well as standards in solvent. Study of possible solutions to correct the matrix effects that do not involve an increase of the analysis time or sample manipulation if feasible.

6. Validation of the developed methods evaluating linearity, accuracy and precision from recovery experiments at different concentration levels. Estimation of the limit of detection and limit of quantification of the methods.

7. Application of the developed analytical methodology to the analysis of different types of water samples (influent, effluent and surface).

8. Confirmation of the identity of the compounds detected in the samples by calculating the ion ratio (intensity of the signal for the selected transitions) in samples and standards.

9. Discussion of the results obtained and establishment of the conclusions related to the presence of pharmaceuticals in the water samples analyzed.

The methodology used for qualitative analysis is the following:

1. Injection of the sample in UHPLC-QTOF and acquisition of the full-spectrum under MS^E acquisition mode at low and high collision energy.

2. Extraction of the exact mass of the protonated (o deprotonated) molecule of every compound from the spectrum at low energy, generating an extracted ion chromatogram (XIC), where the presence of a compound in the sample leads to the corresponding chromatographic peak.

3. Calculation of the experimental error mass by comparison of the accurate mass with the theoretical exact mass.

4. Identification of relevant fragment ions in the spectrum at high energy (if the investigated compound is really present in the sample, the retention time of the chromatographic peaks obtained after performing XICs at the exact masses of the fragments should be the same as for the protonated molecule and should show the same peak shape).

5. Calculation of the mass error of the fragment ions.

6. Deduction of their structures by application of suitable software (MassFragment) or by prediction, taking into account the structural differences with the parent pharmaceutical, and evaluation of the information by means of bibliographic search.

7. Analysis of water samples and investigation of the metabolites/TPs of pharmaceuticals following the developed methodology. Tentative identification on the basis of the information provided by QTOF MS.

8. If commercial reference standards are available, then the injection and comparison of their exact masses, fragment ions and retention times will be made for the unequivocal identification of the metabolite detected in the samples

The **working plan** followed in the Thesis is the following is shown:

1. Selection of the pharmaceuticals to be investigated according to several criteria: most consumed pharmaceuticals in Spain with medical prescription, based on the information provided by the Spanish Ministry of Health; compounds selected according to their potentially negative effects in the living organisms of the aquatic environment; pharmaceuticals detected in surface water and wastewater in studies reported by other authors.

2. Bibliographic revision of the state-of-the-art on the current analysis methods for the determination of pharmaceuticals by LC-MS/MS.

3. Development and optimization of the two multi-residue analytical methods in water for the simultaneous determination of pharmaceuticals with different physico-chemical characteristics belonging to different therapeutical classes. Favouring fast chromatographic analysis by the use of UHPLC. Study of matrix effect and proposals for its correction. Validation of the developed methods.

4. Application of the developed analytical methodology to surface water samples collected from different sites of the Valencian Community and to wastewater samples (influent and effluent) collected from different WWTPs located in the Castellon province.

5. Development, optimization and validation of a method based on UHPLC-MS/MS for the simultaneous determination of pharmaceuticals frequently detected and personal care products. Detailed study of SPE process efficiency and of matrix effects in different types of water. Correction of potential SPE losses and of matrix effects (enhancement/suppression of the signal).

6. Application of the previous method to the analysis of samples from Spain and Colombia in a collaborative study with the Antioquia University.

7. Investigation of pharmaceutical metabolites in effluent wastewater samples previously analyzed by UHPLC-QTOF MS under MS^E mode, using a retrospective analysis of data acquired (exact mass, full spectrum).

8. Use of high resolution mass spectrometry (QTOF MS) for the identification of metabolites of the same parent pharmaceutical that share common fragment ions. Metabolites of the pharmaceutical dipyrone are included in this section, as an illustrative example.

9. Development and validation of a UHPLC-MS/MS method for the quantitative determination of a notable number of metabolites identified in previous analysis by QTOF MS, as well as other metabolites detected in waters according to the scientific literature, along with their parent pharmaceuticals.

10. Application of the UHPLC-MS/MS QqQ analytical methodology developed to the quantitative analysis of pharmaceuticals and metabolites in surface and waste water samples.

11. Elaboration of the main conclusions derived from the research carried out in this Thesis.

Capítulo 1

Introducción general

1.1 Introducción

Los fármacos pertenecen a un amplio grupo de compuestos denominados "contaminantes emergentes". Dichos contaminantes son sustancias químicas de origen natural o sintético cuya presencia en el medio ambiente había pasado desapercibida hasta hace pocos años, bien porque no se habían detectado o porque no se habían reconocido como compuestos de interés desde el punto de vista medioambiental. Se trata pues de un término que presenta una cierta ambigüedad ya que estos contaminantes no son necesariamente sustancias nuevas (Söderström, 2009).

El término contaminantes emergentes engloba a un grupo diverso de compuestos que incluye a los fármacos y productos de cuidado personal, drogas de abuso, algas y toxinas de cianobacterias, retardantes de llama, plastificantes, hormonas y otros compuestos disruptores endocrinos, productos de desinfección, organometálicos, nanomateriales, plaguicidas polares y sus metabolitos (Petrovic, 2010). La Agencia de Protección Medioambiental de los Estados Unidos (EPA, US) revisa periódicamente esta clasificación, incorporando aquellos compuestos que se detectan con frecuencia en las aguas medioambientales. Además de la EPA, otros organismos dedicados a la protección de la salud pública y medioambiental, tales como la Organización Mundial de la Salud (OMS) o la Comisión Europea, consideran que para asegurar la calidad del agua, el estudio de estos compuestos debe ser una línea de investigación prioritaria.

En la actualidad, los contaminantes emergentes no están sujetos a ninguna legislación que regule sus niveles máximos permitidos en el agua. Sin embargo, la alarma creada sobre sus posibles efectos adversos, tanto en el medio ambiente como en

la salud humana, así como su frecuencia en el agua, los ha convertido en posibles candidatos para una futura regulación. Se sabe además que debido a su elevada producción y consumo, y como consecuencia de su continua introducción en el medio ambiente, no necesitan ser persistentes para ocasionar efectos negativos (Petrovic, 2003). Por ese motivo algunos de ellos forman parte de la lista de contaminantes candidatos (*Contaminants Candidate List*, CCL-3) para una posible regulación en aguas de consumo humano, según propuso la EPA en el año 2009. Esta lista incluye contaminantes orgánicos que se encuentran o que se espera que se encuentren en las aguas potables y cuya presencia continua podría ocasionar un cierto riesgo en la salud humana.

En el caso de los fármacos, la Agencia Europea de Medicamentos (EMEA) establece a través de su guía la necesidad de evaluar los riesgos potenciales que los fármacos de uso humano podrían causar en el medio ambiente. Dicha guía señala que antes de comercializar un nuevo producto se debe llevar a cabo una evaluación del riesgo ambiental. Con dicho fin se realizan tests de ecotoxicidad, generalmente a pequeñas escalas de tiempo. Además, la guía indica que también se ha de evaluar otra información que resulta de interés desde el punto de vista ambiental, como es la degradabilidad, el metabolismo, la excreción y la persistencia del compuesto así como la de sus metabolitos.

El aumento en la detección de un gran número de contaminantes emergentes en el agua, algunos de ellos identificados por primera vez en los últimos años, podría llevar a pensar que la calidad del agua ha empeorado. Sin embargo, esta impresión es errónea pues muchos de estos contaminantes han estado presentes en el medio ambiente durante décadas pero, o bien no se analizaban porque no se les consideraba de interés, o bien la falta de sensibilidad de los métodos analíticos impedía su detección (Buchberger, 2011).

Entre los contaminantes emergentes más detectados y cuya presencia ha suscitado mayor interés se encuentran los fármacos. Este grupo de compuestos es el objeto de estudio de la presente Tesis.

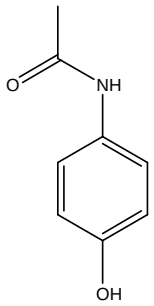
Fármacos

Los fármacos son sustancias químicas que se utilizan para prevenir, curar o aliviar una enfermedad y corregir o reparar las secuelas de ésta. Se estima que unos 3000 compuestos diferentes se utilizan como ingredientes farmacéuticos. Constituyen pues un grupo de compuestos muy amplio y heterogéneo, por lo que existen diversos criterios de clasificación, por ejemplo, en función de su origen, de su estructura o de su mecanismo de acción.

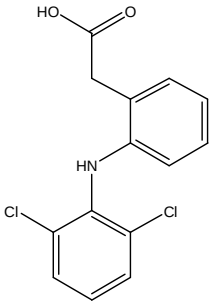
En esta Tesis los fármacos estudiados se han clasificado por grupos terapéuticos, es decir, en función del uso al que se destinan, por ser ésta la clasificación empleada habitualmente en este tipo de trabajos.

Dado que la lista de grupos terapéuticos es muy amplia, en este apartado sólo se hará mención a los principales grupos estudiados en esta Tesis. Las estructuras químicas de los compuestos analizados en los trabajos presentados se muestran a continuación.

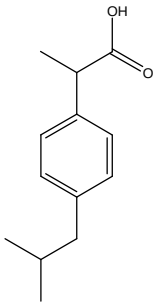
Analgésicos y antiinflamatorios: los fármacos pertenecientes a este grupo se utilizan para eliminar o calmar el dolor, la inflamación y la fiebre gracias a sus propiedades analgésicas, antiinflamatorias y antipiréticas. Se trata de uno de los grupos de compuestos más recetados. Además, también pueden adquirirse sin receta médica. Desde el punto de vista de su estructura son compuestos ácidos débiles, polares y altamente solubles en el medio acuoso por lo que se detectan con frecuencia en el agua (Gros, 2009; Kasprzyk-Hordern, 2009). Dentro de este grupo se encuentran compuestos tan conocidos como el paracetamol (también conocido como acetaminofén), ácido acetilsalicílico, ibuprofeno, metamizol (o dipirona), diclofenaco, ketoprofeno, etc.



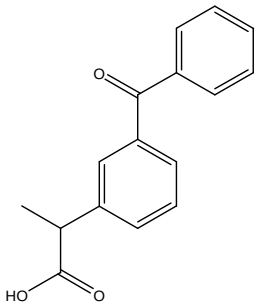
acetaminophen



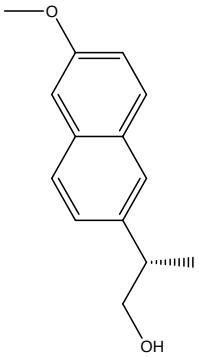
diclofenac



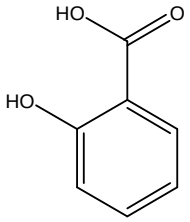
ibuprofen



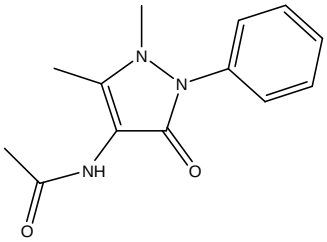
ketoprofen



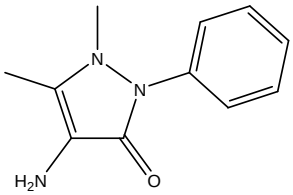
naproxen



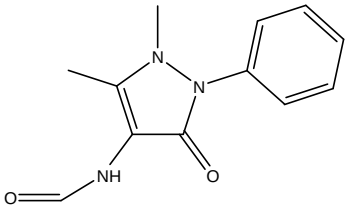
salicylic acid



4-acetamidoantipyrine



4-aminoantipyrine

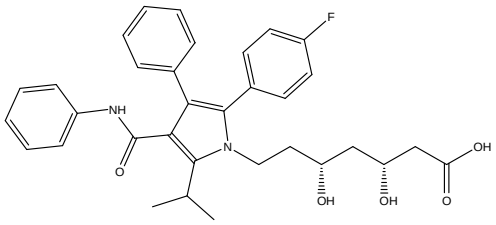


4-formylaminoantipyrine

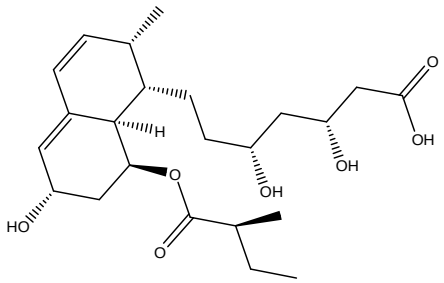
Reguladores lipídicos: este grupo de compuestos se utiliza para disminuir los niveles de colesterol en sangre y para la prevención de enfermedades cardiovasculares. Se distinguen dos tipos de reguladores, los pertenecientes a la clase de los fibratos, por ser derivados del ácido fíbrico, y las estatinas.

Al primer grupo pertenecen, entre otros, el bezafibrato, gemfibrozil, ácido fenofíbrico y ácido clofíbrico. Estos dos últimos son los metabolitos activos del fenofibrato y del clofibrato respectivamente.

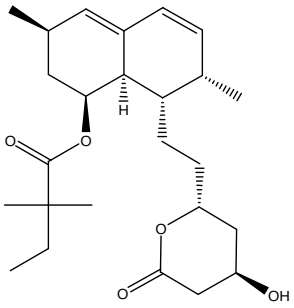
Las estatinas son actualmente uno de los grupos más consumidos. Poseen en su estructura un hidroxiácido de seis miembros similar a la HMG-CoA reductasa, que es la enzima que controla la síntesis de colesterol. Por ese motivo son capaces de actuar como inhibidores competitivos de dicha enzima. Entre las más estudiadas se encuentran la atorvastatina, simvastatina, pravastatina y lovastatina.



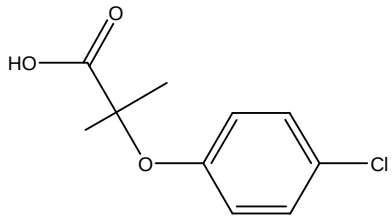
atorvastatin



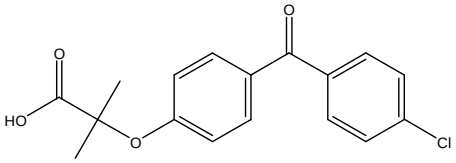
pravastatin



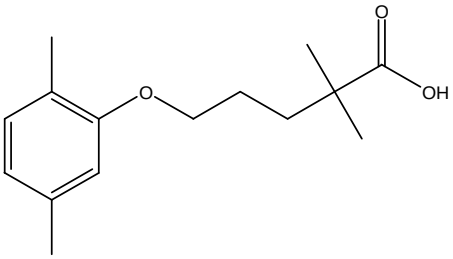
simvastatin



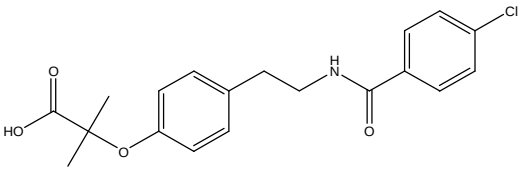
clofibric acid



fenofibric acid



gemfibrozil



bezafibrate

Drogas psiquiátricas: bajo este término se engloba a los fármacos utilizados como ansiolíticos, antipsicóticos, antidepresivos, estimulantes y depresores.

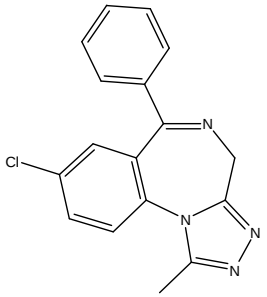
Los *ansiolíticos* son capaces de controlar la ansiedad y el insomnio y de combatir el estrés. Muchos de ellos se utilizan también para el tratamiento de la epilepsia (como relajantes musculares) y para superar la abstinencia del alcohol o de otras drogas adictivas. Pueden ser de dos tipos, los barbitúricos, tales como el amobarbital, pentobarbital, secobarbital, etc., y las benzodiazepinas (diazepam, lorazepam, alprazolam, etc.). Estas últimas aparecieron más tarde cronológicamente y, al ser más seguras, desplazaron a los barbitúricos. Este hecho justifica que los métodos de análisis desarrollados en los últimos años se enfoquen en la determinación de las benzodiazepinas (Baker, 2011). Su estructura química está compuesta por un anillo de benceno unido a otro anillo de siete miembros heterocíclicos llamado diazepina.

Los *antipsicóticos* se utilizan para el tratamiento de la psicosis, enfermedad que consiste en la pérdida de contacto con la realidad. Se trata de un grupo de fármacos

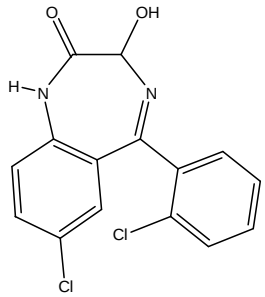
químicamente heterogéneos. Entre los más consumidos destacan la risperidona, olanzapina y quetiapina.

Los *antidepresivos* están indicados, como su propio nombre indica, para el tratamiento de la depresión. Se clasifican en tres grandes grupos: los inhibidores selectivos de la recaptación de la serotonina, los cíclicos y los inhibidores selectivos de la monoaminooxidasa. Los primeros son actualmente los más consumidos (paroxetina, fluoxetina, citalopram, etc.). Se trata de fármacos de origen sintético con una estructura química muy diversa.

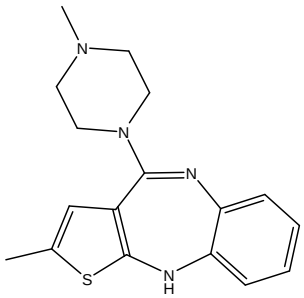
Generalmente se incluye el fármaco carbamazepina dentro del grupo de las drogas psiquiátricas debido a que se utiliza como antidepresivo. Además, también se emplea para el tratamiento de la epilepsia y del trastorno bipolar y como anticonvulsante (Miao, 2003). Del mismo modo, también se incluye la gabapentina, que se utiliza para el tratamiento de la epilepsia y, en menor medida, para el tratamiento de la ansiedad.



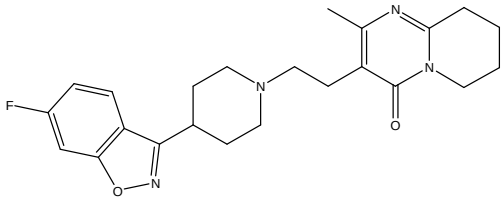
alprazolam



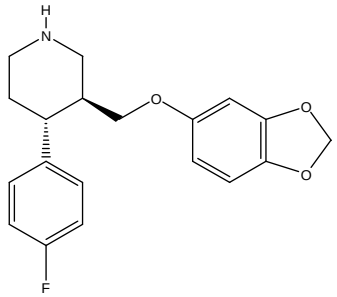
lorazepam



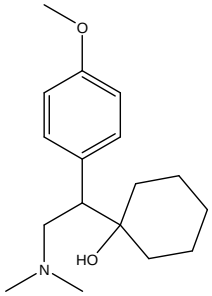
olanzapine



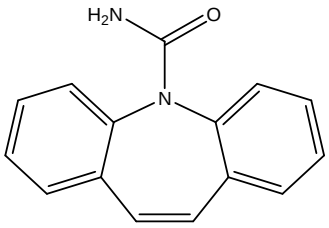
risperidone



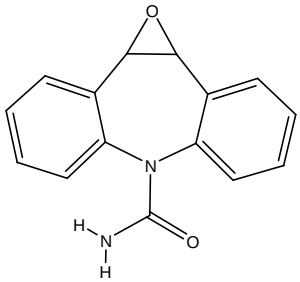
paroxetine



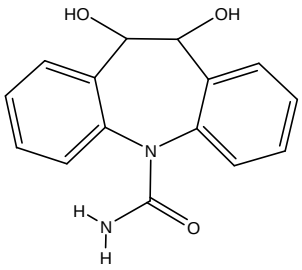
venlafaxine



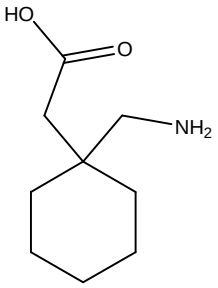
carbamazepine



carbamazepine 10,11-epoxide

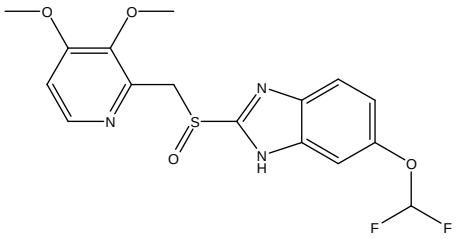


10,11-dihydro-10,11-dihydroxy carbamazepine

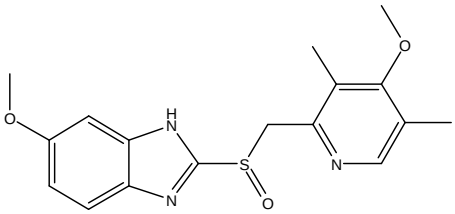


gabapentine

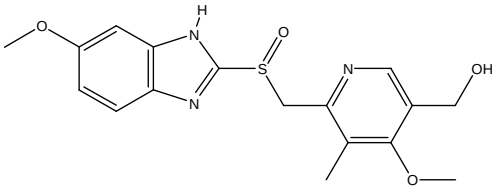
Antiulcerosos: son compuestos que se emplean para prevenir, aliviar los síntomas y la cicatrización de las úlceras del estómago. Según su mecanismo de acción se pueden distinguir cuatro grupos. De ellos destacaremos el grupo al que pertenecen el omeprazol, pantoprazol y lansoprazol por tratarse de los fármacos antiulcerosos más consumidos. Estos compuestos inhiben la secreción gástrica ácida.



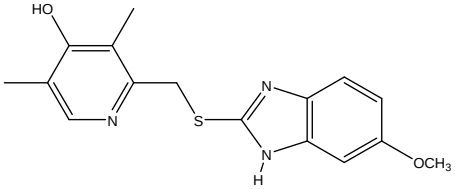
pantoprazole



omeprazole

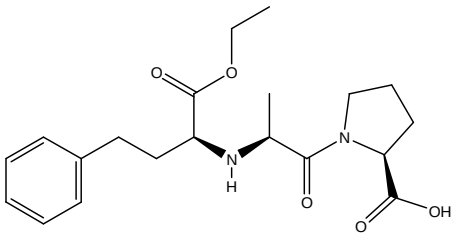


5-hydroxy omeprazole

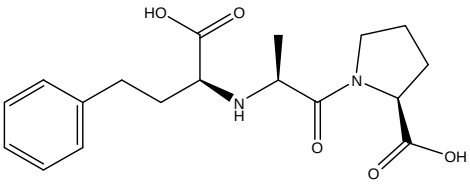


4-hydroxy omeprazole sulphide

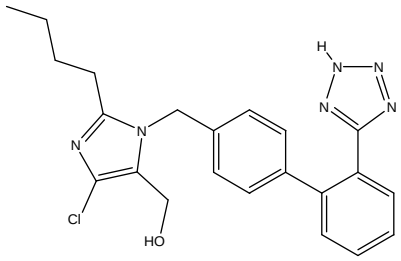
Antihipertensivos: este grupo está integrado por aquellas sustancias que se emplean para reducir la hipertensión arterial. Estos fármacos pueden clasificarse en diversos grupos en función de su mecanismo de acción. Los más utilizados son el enalapril, valsartán y diltiazem.



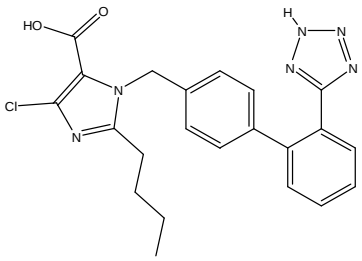
enalapril



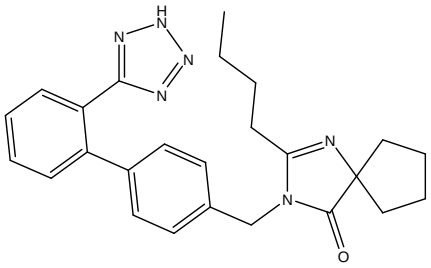
enalaprilat



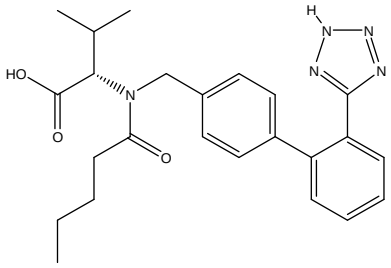
losartan



losartan carboxylic acid



irbesartan



valsartan

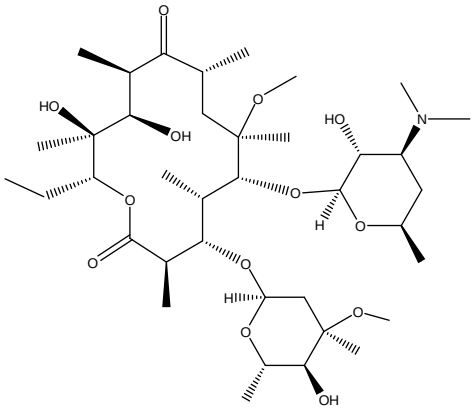
Los antibióticos constituyen uno de los grupos de compuestos cuya presencia en el medio ambiente ha suscitado mayor interés debido a los problemas que podrían originar en los ecosistemas y en la salud humana. Su problemática se comentará con más detalle en el Capítulo 2.

Se definen como compuestos de origen natural, semisintético y sintético con actividad antibacteriana, antifungicida o antiparasitaria (Kümmerer, 2009). Se emplean para el tratamiento de las infecciones tanto en las personas como en los animales. Se utilizan también en la agricultura para controlar las enfermedades bacterianas durante el crecimiento de la fruta y de los vegetales, y en la ganadería para promover el crecimiento. Cabe mencionar que, en el último caso, la Unión Europea prohibió esta práctica en el año 2006 (Reglamento 1831/2003).

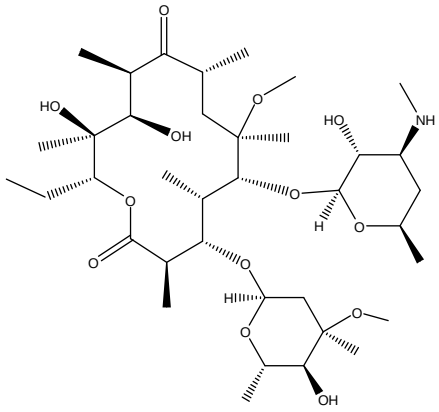
Los antibióticos son sustancias producidas por varias especies de microorganismos (bacterias u hongos) que al actuar sobre otros microorganismos son capaces de suprimir su crecimiento y multiplicación (acción bacteriostática) o de provocar su destrucción (acción bactericida). Actualmente se ha extendido el término de antibiótico a los agentes antibacterianos sintéticos como son las sulfonamidas y las quinolonas.

Atendiendo a su estructura química, los antibióticos se clasifican en familias. Dada la amplia clasificación que existe en este apartado sólo se hará mención a aquellas familias estudiadas en esta Tesis.

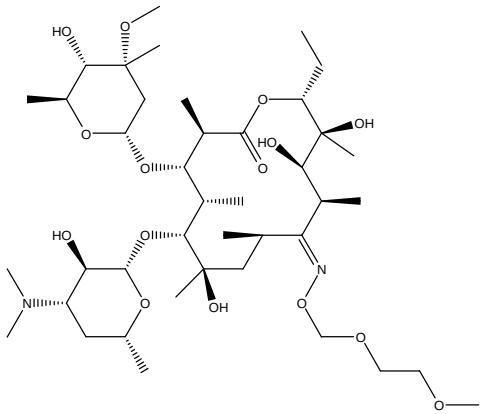
Los *macrólidos* se usan tanto en medicina humana como en veterinaria. Se trata de compuestos básicos lipofílicos que se caracterizan por tener un anillo macrocíclico de lactona con 14, 15 ó 16 átomos y azúcares unidos por enlaces glucosídicos. La eritromicina fue el primer macrólido descubierto y hoy en día sigue siendo uno de los más importantes. La claritromicina, roxitromicina y azitromicina son derivados semisintéticos de la eritromicina.



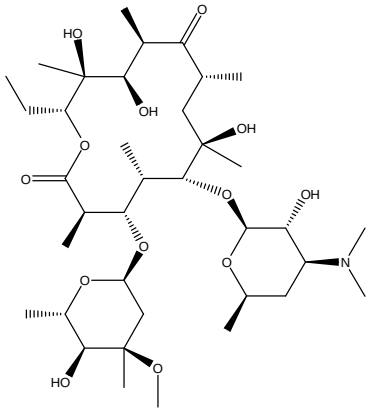
clarithromycin



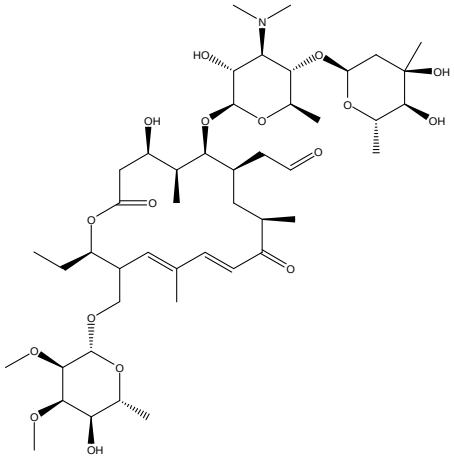
N- desmethyl clarithromycin



roxithromycin



erithromycin



tylosin

Las *quinolonas* tienen una estructura formada por dos anillos, con un nitrógeno en la posición 1, un grupo carbonilo en la posición 3 y un grupo carboxilo en la posición 4. Si poseen un átomo de flúor en la posición 6 aumenta su potencia y se les llama fluoroquinolonas. También aumenta su potencia si en la posición 7 hay un grupo piperacínico (norfloxacino, ciprofloxacino) o un grupo metil-piperacínico (ofloxacino) (Alós, 2009).

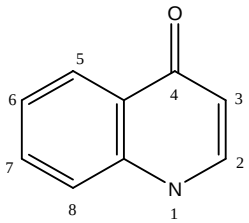
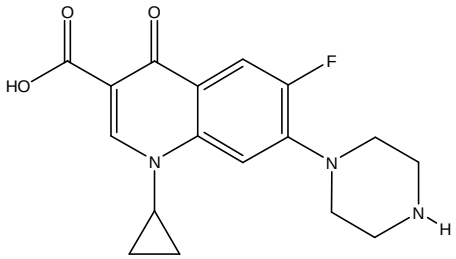
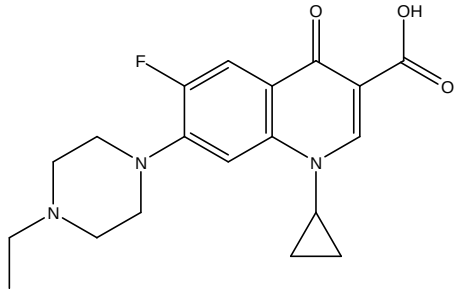


Figura 1.1 Estructura de la 4-quinolona, molécula de donde derivan muchas de las quinolonas utilizadas.

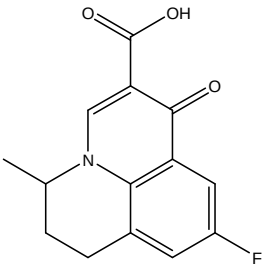
Las quinolonas actúan inhibiendo la síntesis del ADN cromosómico bacteriano y actualmente se usan para el tratamiento de una gran variedad de infecciones. Desde que se introdujo el uso del ácido nalidíxico en los años sesenta del siglo pasado se han desarrollado un número considerable de quinolonas que pueden clasificarse en cuatro generaciones según el espectro sobre el que actúan.



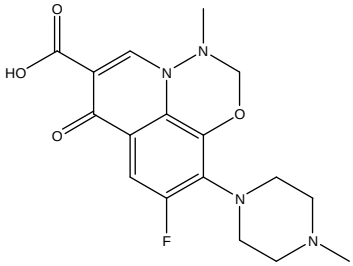
ciprofloxacin



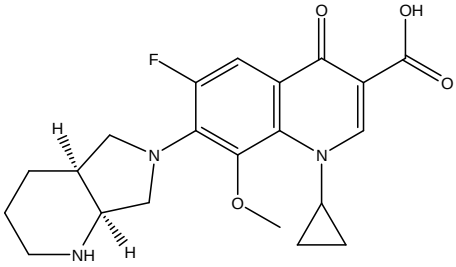
enrofloxacin



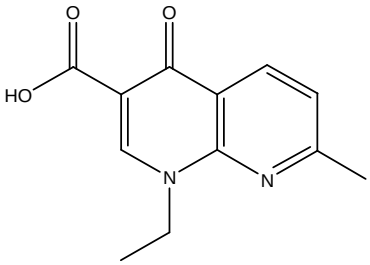
flumequine



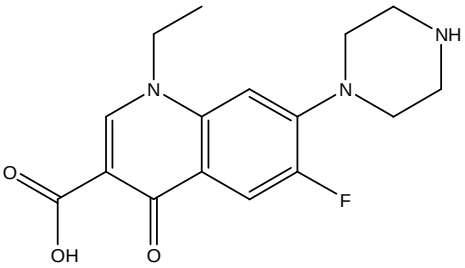
marbofloxacin



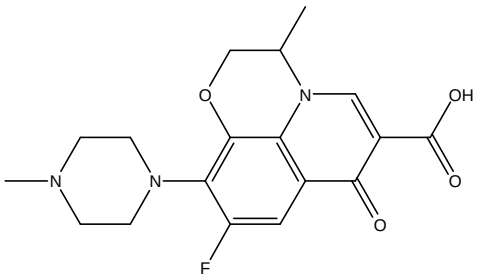
moxifloxacin



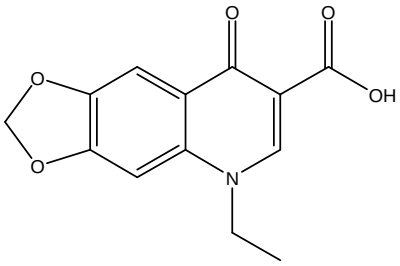
nalidixic acid



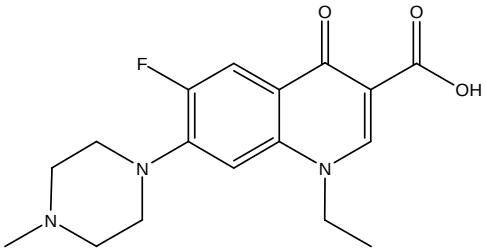
norfloxacin



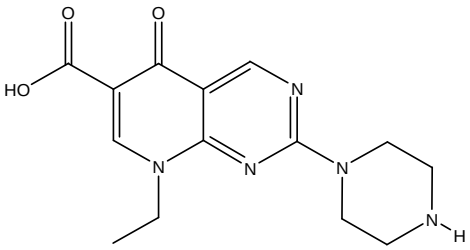
ofloxacin



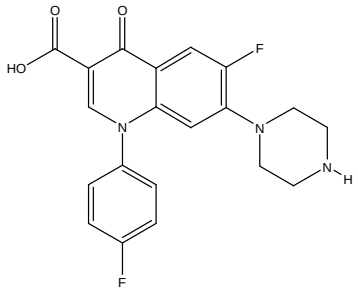
oxolinic acid



pefloxacin

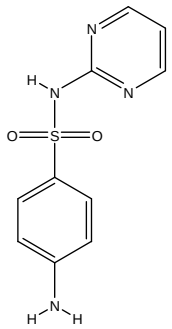


pipemidic acid

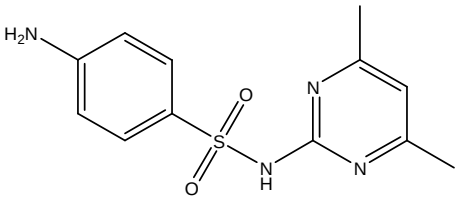


sarafloxacin

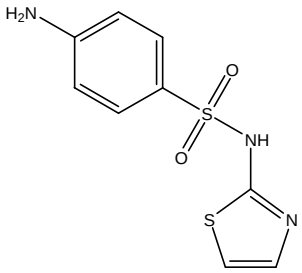
El término *sulfonamida* se ha utilizado como nombre genérico para designar a los derivados del 4-aminobencenosulfonamida (sulfanilamida). Estos compuestos actúan inhibiendo el crecimiento y reproducción de las bacterias. Entre las sulfonamidas, sulfametoxazol es una de las más detectadas en las muestras procedentes de las Estaciones Depuradoras de Aguas Residuales (EDAR) (Le-Minh, 2010).



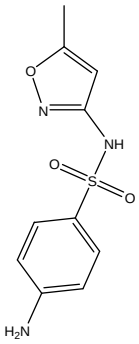
sulfadiazine



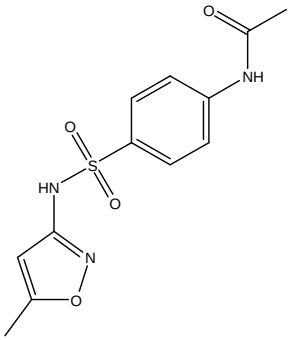
sulfamethazine



sulfathiazole

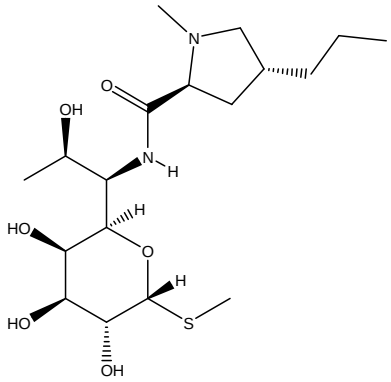


sulfamethoxazole

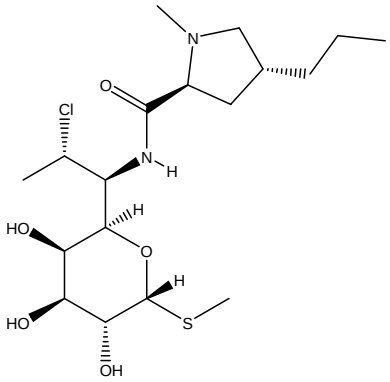


N-acetyl sulfamethoxazole

La lincomicina y su derivado, la clindamicina, son las dos *lincosamidas* disponibles. Al sustituir un grupo hidroxilo por un átomo de cloro se obtiene la clindamicina. Este compuesto posee mayor actividad y por ello la lincomicina está actualmente en desuso.

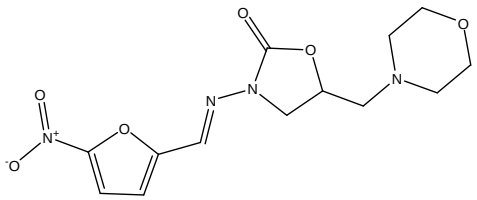


lincomycin

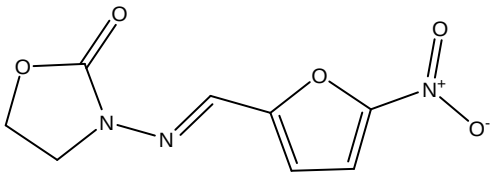


clindamycin

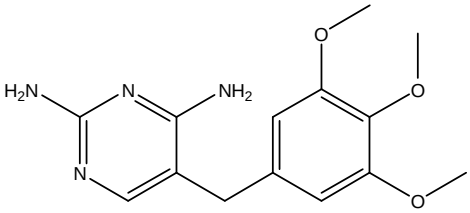
Otros fármacos estudiados:



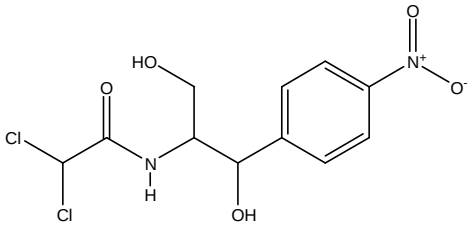
furaltadone (antibiótico)



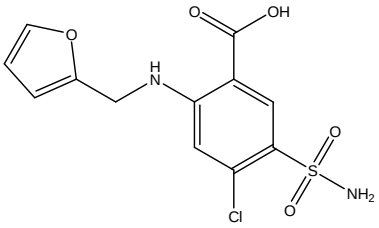
furazolidone (antibiótico)



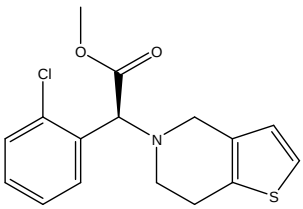
trimethoprim (antibiótico)



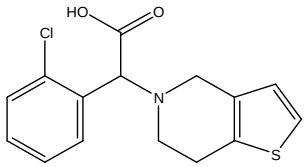
chloramphenicol (antibiótico)



furosemide (diurético)



clopidogrel (agente antiplaquetario)



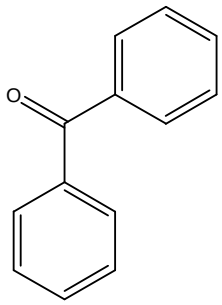
clopidogrel carboxylic acid

Productos de cuidado personal

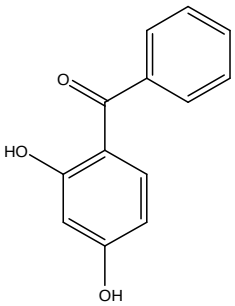
El término productos de cuidado personal engloba a los compuestos orgánicos utilizados en los productos de belleza y de higiene, como los geles, lociones, cremas, perfumes, pasta de dientes, etc.

Estos compuestos se clasifican en cinco grupos en función de su uso (Pedrouzo, 2011).

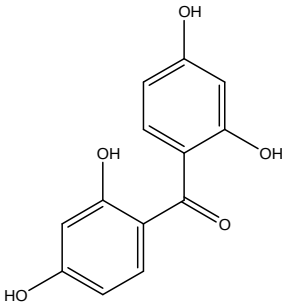
Filtros orgánicos ultravioleta: se utilizan principalmente en los cosméticos de protección solar y en otros productos para la protección de la radiación ultravioleta (UV). Se caracterizan por la presencia de uno o varios anillos aromáticos, generalmente con grupos hidrofóbicos unidos. Los filtros ultravioleta más detectados en el agua son las benzofenonas.



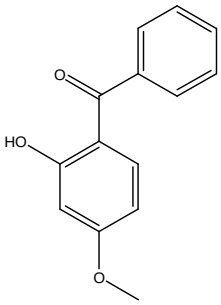
benzophenone



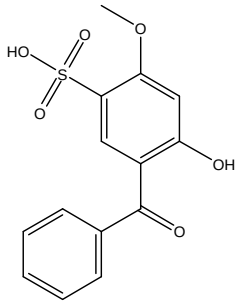
benzophenone-1



benzophenone-2

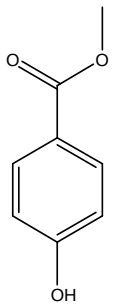


benzophenone-3

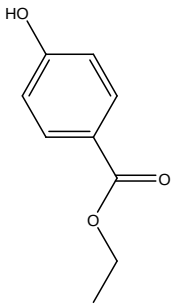


benzophenone-4

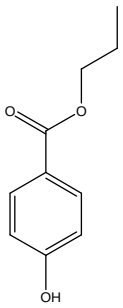
Preservantes: se utilizan para evitar el deterioro y, en consecuencia, para aumentar el tiempo de caducidad de una gran variedad de productos empleados en la vida cotidiana, tanto cosméticos como comestibles. Los parabenos son los más empleados.



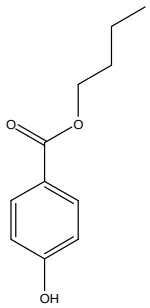
methylparaben



ethylparaben



propylparaben



butylparaben

Antimicrobianos: son los compuestos que pueden matar o prevenir el crecimiento de microbios, tales como las bacterias, virus, hongos o parásitos. Los más consumidos son el triclosán y triclocarbán.

Fragancias de almizcle: son compuestos químicos artificiales muy utilizados en una gran cantidad de productos perfumados de belleza y de limpieza. Existen tres grupos de fragancias sintéticas llamadas nitro almizcles, almizcles policíclicos y macrocíclicos.

Repelentes de insectos: son compuestos químicos que, aplicados sobre la piel, impiden que los insectos se fijen y realicen una picadura. Los principales repelentes empleados en las formulaciones químicas son *N*,*N*-Dietil-meta-toluamida (DEET) y, más recientemente, el compuesto butil 2-(2-hidroxietil)-1-piperidin carboxilato, conocido comercialmente como Bayrepel®.

Los productos de cuidado personal suelen analizarse junto con los fármacos. Por ese motivo, los apartados que se presentan a continuación son comunes para ambos tipos de contaminantes.

1.2 Investigación de fármacos en muestras de agua. Metodología analítica

La presencia de los fármacos en el medio ambiente es un campo de estudio reciente. Durante las últimas décadas los investigadores han centrado su atención en el estudio de contaminantes conocidos por su toxicidad y persistencia en el medio ambiente. Se trata, en su mayoría, de compuestos regulados por la legislación. Este es el caso de los hidrocarburos aromáticos policíclicos y de las dioxinas, entre otros. En cambio, la mejora de la sensibilidad de las técnicas de detección utilizadas ha permitido identificar compuestos cuya presencia hasta ahora había pasado desapercibida. Este es el caso de los fármacos.

El primer informe sobre la presencia de residuos de fármacos en el medio ambiente fue publicado por Garrison en el año 1976, de la Agencia de Protección Medioambiental de los Estados Unidos de América. En dicho estudio se identificó la presencia de ácido clofíbrico (el metabolito activo de varios reguladores lipídicos) y de ácido salicílico en las aguas de una EDAR. Sin embargo, esta publicación no supuso el inicio inmediato de una nueva línea de investigación ya que tras ella sólo se realizaron algunos estudios aislados (Richardson, 1985). A principios de la década de los 90, en un estudio dedicado a la determinación de la presencia de residuos de plaguicidas en agua subterránea y agua para consumo humano, se detectó un compuesto desconocido que resultó ser ácido clofíbrico (Stan, 1992). A partir de ese momento la presencia de los fármacos en el medio ambiente atrajo la atención de los investigadores. Desde entonces hasta la actualidad un gran número de estudios publicados han confirmado la existencia de una amplia variedad de fármacos en las aguas medioambientales y residuales, en sedimentos y en lodos.

En los primeros trabajos realizados la cromatografía de gases acoplada a la espectrometría de masas (GC-MS) fue la técnica utilizada para la determinación de fármacos (Ternes, 1998). Esta técnica resulta adecuada para el análisis de compuestos no polares y compuestos volátiles por lo que, para poder aplicarla al análisis de compuestos polares, se requiere una etapa previa de derivatización de la muestra

utilizando agentes derivatizantes (ej. diazometano, agentes silanizantes, anhídridos de ácidos) (Wille, 2012). Se trata de una etapa compleja que requiere mayor tratamiento de muestra y un proceso previo de optimización (Fatta-Kassinos, 2011) y que conlleva un mayor tiempo de análisis (Gros, 2006). Debido a ello, tras al aparición del acoplamiento LC-MS y LC-tándem MS (LC-MS/MS), esta técnica se convirtió en la preferida para el análisis de los compuestos orgánicos polares, como es el caso de los fármacos.

Introducción de los fármacos en el medio ambiente

Los fármacos son compuestos ampliamente utilizados en la medicina humana y veterinaria. Pueden llegar al medio ambiente a través de diversas vías de contaminación, siendo la principal la de su ingestión y posterior excreción.

El compuesto ingerido puede sufrir una transformación en el cuerpo humano dando lugar a sustancias más solubles y polares, conocidas como metabolitos, o unirse a otras moléculas para formar un compuesto conjugado. Posteriormente, los compuestos ingeridos inalterados y/o sus metabolitos se excretan en la orina y heces llegando a las EDAR a través de las aguas residuales domésticas. En el caso de los productos de cuidado personal, llegan al agua directamente después de lavarse la piel y la ropa, o indirectamente a través de las aguas residuales o del agua de las piscinas (Fent, 2010).

Actualmente, los tratamientos de depuración empleados en las EDAR no son capaces de eliminar por completo la mayoría de estos compuestos y, en consecuencia, se vierten a las aguas medioambientales a través de los efluentes.

Por otro lado, la eliminación de los fármacos caducados o que no se utilizan a través del inodoro o de la basura doméstica también contribuye a la contaminación del medio ambiente, aunque en mucha menor medida que vía su excreción (Gros, 2006; Nikolaou, 2007).

Además de estas rutas de contaminación, los fármacos empleados con uso veterinario y como aditivos para piensos en la cría de ganado pueden llegar al suelo a través del estiércol. Tras la lluvia pueden pasar a las aguas superficiales por la escorrentía de los campos tratados con estiércol o con lodos tratados procedentes de las depuradoras que se utilizan como biosólidos (Petrovic, 2003; Nikolaou, 2007).

En la Figura 1.2 se muestra un esquema en el que señalan las principales vías de contaminación de los fármacos en el medio ambiente.

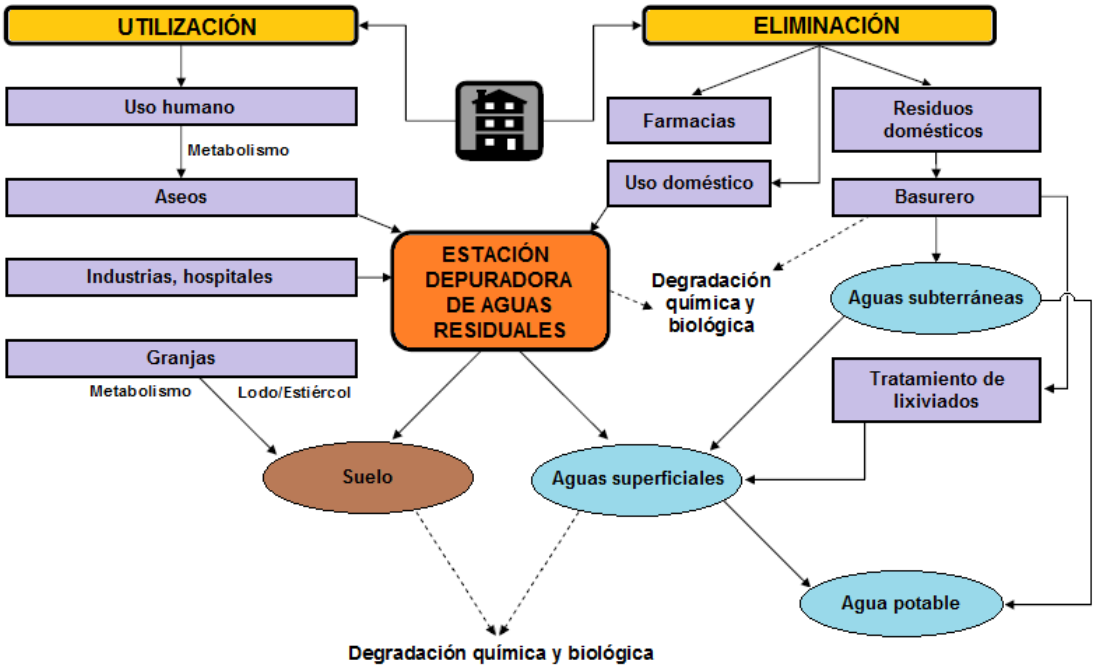


Figura 1.2 Esquema general de la llegada de los fármacos al medio ambiente. Modificado de Nikolaou, 2007.

Tras alcanzar el medio ambiente mediante cualquiera de las vías anteriormente mencionadas pueden experimentar procesos de degradación. En la mayoría de las ocasiones estos procesos consisten en reacciones de hidrólisis, redox y, especialmente, fotólisis. Esta última origina la degradación de los compuestos mediante la absorción de la radiación solar a través alguno de los grupos funcionales fotosensibles de la molécula (ej. anillos aromáticos, heteroátomos).

Los fármacos también pueden experimentar procesos de biodegradación, es decir, descomponerse mediante la acción de organismos vivos presentes en el agua y en el suelo. Sin embargo, al tratarse de compuestos biológicamente activos diseñados para ser resistentes a la biodegradación, les convierte en sustancias persistentes en el medio ambiente (Fatta-Kassinos, 2011).

Se trata de compuestos que tienen una vida media larga en el medio ambiente. Por ese motivo pueden acumularse alcanzando niveles detectables y biológicamente activos. Algunos de ellos son persistentes mientras que otros son pseudo persistentes, es decir, aunque se degradan en el medio ambiente a un ritmo razonable, se reemplazan continuamente debido a su uso generalizado (Khetan, 2007).

Aunque en la actualidad existe abundante bibliografía sobre la presencia de los fármacos en el medio ambiente, en realidad se sabe poco a cerca de sus posibles efectos en el medio ambiente, especialmente en el caso de mezclas de estos compuestos.

1.2.1 Tratamiento de muestra

El análisis de fármacos en el agua, así como el de otros compuestos orgánicos, resulta especialmente complicado por la complejidad de las matrices analizadas y por los bajos niveles de concentración en los que se encuentran. Por ese motivo, la gran mayoría de los métodos utilizados en la actualidad incluyen una etapa de extracción, tanto para la limpieza como para el enriquecimiento de las muestras acuosas.

El tratamiento de muestra más utilizado para alcanzar la sensibilidad deseada es la extracción en fase sólida (SPE) en modo *off-line*. Esta técnica se considera la más versátil y fiable ya que permite ajustar el volumen del agua extraída (generalmente, entre 50 y 1000 mL) y las condiciones de elución para obtener la sensibilidad deseada y maximizar las recuperaciones de los compuestos (Bagnati, 2011).

En el caso de matrices complejas, como las aguas residuales procedentes de una depuradora, se requiere una etapa de filtración o de centrifugación previa a su

extracción. De este modo se consigue eliminar partículas en suspensión que podrían obstruir el cartucho.

Previamente a la extracción de la muestra se puede ajustar su pH o añadir ciertos reactivos para mejorar la eficacia de la extracción. Los reactivos más utilizados son los agentes quelantes y, entre ellos, el ácido etilendiaminotetraacético (EDTA). Estos compuestos tienen la propiedad de combinarse con algunos metales para formar complejos. Así se evita que dichos metales se unan a algunos analitos, en especial a las tetraciclinas. El ajuste del pH de la muestra puede aumentar la afinidad de los analitos por el sorbente SPE (Wong, 2009), como se comentará a continuación.

Los sorbentes SPE más utilizados para la extracción de los fármacos en matrices acuosas son los poliméricos. Los cartuchos de sílica C18 también se emplean, pero en menor grado.

Los sorbentes poliméricos tradicionales se basan en el uso de materiales en fase reversa como la sílice modificada con cadenas de alquil o divinilbenceno poliestiereno. Este tipo de material es adecuado para los fármacos que poseen una cierta hidrofobicidad pero no lo es tanto para aquellos compuestos con propiedades polares. Para estos casos es posible encontrar una alternativa basada en sorbentes que han incorporado grupos funcionales en la estructura polimérica, dando lugar a sorbentes de fase reversa con balance hidrofílico-lipofílico. Este tipo de sorbentes permiten la retención de compuestos con un amplio rango de polaridades. Entre ellos, el sorbente Oasis HLB se ha convertido en el más utilizado en el análisis de fármacos en muestras medioambientales, sobre todo en el caso de métodos multirresiduales, ya que permite extraer simultáneamente analitos ácidos, básicos y neutros.

A partir de la estructura de este sorbente se han desarrollado otros que presentan una alta selectividad para compuestos básicos y ácidos mediante la adición de grupos intercambiadores de cationes (grupos ácido sulfónico) y grupos intercambiadores de aniones (grupos amina cuaternaria) respectivamente. En el primer caso se trata de los sorbentes Oasis MCX y en el segundo, de los Oasis MAX. En ambos tipos de sorbentes los analitos se retienen mediante interacciones iónicas mientras que

el resto de los componentes de la matriz lo hacen a través de la fase reversa. Estos sorbentes requieren un ajuste adecuado del pH de la muestra así como del disolvente de elución, mientras que los sorbentes del tipo HLB permiten trabajar en un amplio rango de pH.

Algunos métodos incluyen una etapa de limpieza del cartucho (habitualmente conocida con el término inglés *clean-up*) tras el paso de la muestra para conseguir separar los analitos de interés de otros compuestos presentes en la matriz que pueden interferir posteriormente en el análisis. El objetivo de esta etapa es conseguir que tan sólo los compuestos de interés queden retenidos en el sorbente.

En cuanto a los disolventes de elución, los más utilizados son los disolventes polares como el metanol, acetona y acetonitrilo.

La SPE también puede realizarse en modo *on-line*, que consiste en un proceso automatizado, es decir, mediante conexión directa del sorbente con el sistema LC-MS, donde tiene lugar la separación cromatográfica. Esta modalidad presenta ciertas ventajas respecto a la *off-line* previamente comentada, tal como la reducción del volumen de muestra y del consumo de disolvente orgánico, una menor manipulación de la muestra y, en consecuencia, minimización del tiempo de análisis requerido. En contra, los métodos de extracción *on-line* pueden presentar una menor sensibilidad (debido a que el volumen de muestra que se puede cargar es menor), experimentar mayores efectos matriz o un peor comportamiento cromatográfico (Bagnati, 2011).

Aunque mucho menos utilizados en este campo que la extracción en fase sólida, existen otros tipos de tratamientos de muestra como son la extracción líquido-líquido, la microextracción (en fase líquida o en fase sólida), extracción por adsorción con barras magnéticas agitadoras (*stir bar sorbent extraction*, SBSE) o los polímeros molecularmente impresos (*molecularly imprinted polymers*, MIPs).

La extracción líquido-líquido fue la técnica de extracción empleada en los primeros trabajos (Garrison, 1976), pero hoy en día ha quedado obsoleta. Se trata de un

procedimiento muy laborioso, lento y que generalmente requiere del uso de grandes cantidades de disolvente.

La microextracción es una técnica de tratamiento de muestra que consiste en que los analitos se adsorben en una fase estacionaria y posteriormente se desorten térmicamente o mediante disolventes (Wong, 2009). La microextracción puede ser en fase sólida (SPME) y en fase líquida (LPME).

La microextracción presenta ciertas ventajas respecto a SPE: el volumen de muestra empleado es mínimo (generalmente, varios mililitros), la cantidad de disolvente utilizada es menor, es un proceso rápido y, además, las etapas de extracción de los analitos, la de limpieza y la de concentración se producen simultáneamente. En contra, la microextracción es menos sensible y menos precisa que otras técnicas debido en gran medida a que muchos de los parámetros implicados se optimizan manualmente.

La SPME se basa en la extracción de los analitos de la matriz mediante una fibra que está recubierta de un sorbente. Si se acopla a GC, la desorción se realiza térmicamente mientras que si se combina con LC se lleva a cabo mediante el uso de un disolvente orgánico. Esta técnica se ha utilizado principalmente para la extracción de analitos apolares ya que hasta el momento se han comercializado pocas fases estacionarias polares. En el caso de analitos polares generalmente se requiere una derivatización. El proceso de optimización es bastante tedioso. Por ejemplo, si la SPME se acopla a LC, la fibra, el pH de la muestra, la temperatura, y el tiempo de extracción y de desorción son, entre otros, los parámetros que se deben optimizar (Lock, 1999; McClure, 2007; Wong, 2009).

En el caso de LPME, los analitos se extraen en una fibra hueca porosa impregnada con una fase líquida inmiscible (por ejemplo, un disolvente orgánico) que recoge los analitos y que posteriormente se analiza. Esta técnica es rápida, sencilla y utiliza tan sólo unos pocos microlitros de disolvente. Además, permite alcanzar bajos niveles de detección como consecuencia de los elevados factores de preconcentración que pueden alcanzarse (Wong, 2009; Buchberger, 2011).

Otra técnica de extracción, muy relacionada con la SPME, es la extracción por adsorción con barras magnéticas agitadoras (SBSE). En este caso el volumen del sorbente de la fase estacionaria es mayor. De este modo, la proporción del analito que se transfiere es mayor que en la SPME y en consecuencia, la sensibilidad es más elevada. Al igual que la SPME, esta técnica se desarrolló para el análisis de analitos no polares que tras adsorberse en la barra se transfieren mediante desorción térmica al sistema GC. El análisis de los analitos polares conlleva una cierta dificultad ya que se debe realizar una derivatización previa, o bien utilizar fases estacionarias muy específicas. Estos inconvenientes justifican que, a día de hoy, el uso de esta técnica de extracción tenga una aplicación limitada.

Otra alternativa, aunque mucho menos utilizada en la actualidad, consiste en el uso de polímeros molecularmente impresos (MIPs). Grosso modo, se basa en sorbentes que poseen unas cavidades específicas diseñadas para unas moléculas molde (o *template molecules*, si se hace uso de la denominación anglosajona) determinadas. Se trata por tanto de una técnica que ofrece una elevada selectividad y sensibilidad pues el sorbente reconoce la forma y los grupos funcionales de la molécula específica para la que se ha diseñado, incluso en presencia de otros compuestos con una estructura y funcionalidad similar. Por ese motivo, la posibilidad de coextraer otros compuestos presentes en la matriz se reduce. Esta propiedad se traduce en una reducción del efecto matriz en comparación con las técnicas de extracción anteriormente explicadas y, en consecuencia, una mayor sensibilidad (Wille, 2012). Esta técnica resulta muy costosa tanto desde el punto de vista económico como del tiempo que supone la elaboración del polímero. Además, no permite llevar a cabo la extracción simultánea de compuestos pertenecientes a distintos grupos terapéuticos (métodos multiclase).

Tal y como afirman algunos autores (Seifrtova, 2009; Buchberger, 2011), en el futuro la tendencia general será la inyección directa de las muestras en el sistema LC con masas en tándem (MS/MS), es decir, no se requerirá una etapa de tratamiento de muestra, o a lo sumo, ésta consistirá únicamente en la filtración o en el ajuste de pH. Esta tendencia se generalizará a medida que se desarrollen equipos cada vez más sensibles.

1.2.2 Análisis mediante cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS)

Dada la complejidad de las matrices medioambientales y los bajos niveles a los que generalmente se encuentra los fármacos, la espectrometría de masas es la técnica que ofrece una mayor sensibilidad y especificidad para la detección de estos compuestos. Por ese motivo la técnica más empleada es LC-MS/MS.

Actualmente la interfase más utilizada en el análisis de fármacos es la de ionización por electrospray (ESI). La interfase de ionización química a presión atmosférica (APCI) hasta ahora se ha aplicado en un número de trabajos más reducido.

La interfase ESI permite analizar sustancias termolábiles dentro de un amplio rango de pesos moleculares, mientras que la APCI está limitada a moléculas con bajos pesos moleculares y no termolábiles. La primera resulta más adecuada para los analitos iónicos y polares. Por el contrario, la interfase APCI ofrece mejores resultados para el análisis de los analitos menos polares. En el caso de los fármacos, al tratarse de compuestos con una polaridad intermedia, se podrían utilizar ambas interfases aunque, por lo general, la interfase ESI permite realizar determinaciones más sensibles.

En todos los trabajos presentados en esta Tesis se ha hecho uso de la interfase ESI, cuyo fundamento se explicará someramente a continuación.

En las interfases ESI el flujo procedente de la LC pasa a través de un capilar a presión atmosférica sometido a un alto voltaje (3-5 kV, con polaridad positiva o negativa dependiendo si se producen iones positivos o negativos). El voltaje aplicado dispersa la corriente del líquido, formando un spray de gotas cargadas (nebulización). Dichas gotas se evaporan a aproximadamente 120 ºC (desolvatación) al atravesar la región a presión atmosférica de la fuente del espectrómetro de masas. La desolvatación es asistida por una corriente de nitrógeno a 350 ºC aproximadamente. El tamaño de las gotas disminuye hasta que las fuerzas repulsivas entre cargas en la superficie superan las fuerzas cohesivas de tensión superficial y se produce la ruptura de la gota, originando iones en fase gaseosa. Este proceso se conoce como “explosión de

Coulomb". Finalmente, los iones formados se transfieren al espectrómetro de masas a través de lentes focalizadoras.

Una de las ventajas de la espectrometría de masas en tándem es que no se requiere una separación cromatográfica completa de los analitos para poder detectarlos selectivamente. Sin embargo, resulta aconsejable tener una buena separación para evitar los problemas de efecto matriz (Gros, 2006-b), que se comentarán con detalle en el apartado 1.2.4, y para evitar identificaciones erróneas en algunas situaciones complejas, como por ejemplo, cuando se investigan metabolitos (Ibáñez, 2012).

Los métodos analíticos más recientes utilizan columnas cortas (generalmente de 5 ó 10 cm de longitud) para reducir el tiempo de análisis. La mayoría de ellos emplean columnas de fase reversa, usando HPLC o UHPLC. Las más utilizadas son las que contienen fases estacionarias con largas cadenas hidrocarbonadas compuestas por 18 átomos de carbono (C18).

Los disolventes orgánicos utilizados en la composición de la fase móvil deben ser polares porque favorecen la ionización de los analitos en la interfase, siendo los más usados metanol y acetonitrilo. La adición de modificadores (también llamados aditivos) puede mejorar la eficacia de la ionización de los compuestos y, en consecuencia, aumentar su sensibilidad. Además, el uso de modificadores también puede mejorar la forma de pico de los compuestos cromatográficos, favorecer su retención en la columna y mejorar la resolución. Generalmente, en el caso de los analitos ácidos, que se determinan en modo negativo, se utilizan disolventes orgánicos y agua HPLC sin modificadores o se añade acetato amónico a la fase acuosa. En cambio, el uso de modificadores que proporcionan un pH neutro o ácido favorece la detección de los compuestos neutros o básicos que se analizan en modo positivo (Gros, 2006). Los modificadores se utilizan a concentraciones relativamente bajas y controladas ya si se adicionan a concentraciones elevadas la intensidad de la señal podría verse afectada.

En los últimos años se ha extendido el uso de una nueva técnica de separación conocida como cromatografía líquida de ultra resolución (UHPLC). Mediante el uso de columnas con un tamaño de partícula menor que las utilizadas en la cromatografía

convencional (diámetro menor de 2 μm frente al tamaño habitual de 5 μm), el empleo de UHPLC proporciona una mayor resolución y sensibilidad gracias a la obtención de picos cromatográficos más estrechos y de mayor altura. Además, la separación cromatográfica es más rápida. Esta técnica requiere el uso de sistemas de detección basados en espectrometría de masas con altas velocidades de barrido, capaces de trabajar con tiempos de monitorización (*dwell times*) bajos. Asimismo, es necesario disponer de bombas especiales que puedan soportar las presiones que pueden generarse en el sistema (por ejemplo, 15000 psi/1000 bares).

Todos los trabajos que se presentan en esta Tesis se han realizado utilizando la técnica UHPLC acoplada a la espectrometría de masas en tándem (UHPLC-MS/MS), que se basa en la combinación de varios analizadores en el tiempo o en el espacio. Se ha hecho uso de los analizadores de triple cuadrupolo (QqQ) e híbrido cuadrupolo-tiempo de vuelo (QTOF), que se describirán a continuación.

El analizador de triple cuadrupolo consiste en dos analizadores cuadrupolares separados entre sí por un hexapolo que actúa como celda de colisión. Algunos de los analizadores más modernos, como el empleado en los trabajos presentados en esta Tesis, no emplean una celda de colisión hexapolar. En su lugar se utiliza un dispositivo denominado *T-Wave* que consiste en una serie de cilindros por donde se aplica una corriente en forma de onda. De esta manera, permite trabajar a mayores velocidades de barrido y, gracias a ello, mantener la sensibilidad incluso a bajos *dwell times* (3 ms). Cuando el ión seleccionado (ion precursor) colisiona con las moléculas de gas inerte (argón) en la celda de colisión, se produce la fragmentación del ión. Este proceso recibe el nombre de disociación inducida por colisión (*collision induced dissociation*, CID). Los iones obtenidos pasan al tercer cuadrupolo donde se puede realizar un barrido de los iones o seleccionar uno de ellos, consiguiendo una mayor selectividad.

Los analizadores de triple cuadrupolo ofrecen múltiples opciones de trabajo en función del objetivo final del análisis. En modo MS el triple cuadrupolo trabaja como un solo analizador cuadrupolar. Esta configuración permite seleccionar dos modos de trabajo: el *full scan* y *Selected Ion Monitoring* (SIM). En el primer caso se realiza un

barrido de todos los iones de un rango de masas definido, mientras que en el modo SIM se selecciona un ión concreto para ser monitorizado. Trabajando en modo MS, estos analizadores presentan una baja sensibilidad. Además, al no haber apenas fragmentación, la información que proporcionan resulta insuficiente para poder asegurar que un cierto compuesto se encuentra presente en una muestra debido al elevado número de moléculas que pueden compartir una misma masa nominal.

Cuando se trabaja en modo MS/MS se puede realizar un barrido de los iones producto obtenidos al fragmentar una determinada m/z, un barrido de los iones precursores, búsqueda de pérdidas neutras, o trabajar en modo *Selected Reaction Monitoring* (SRM). Este modo de trabajo es el que se ha utilizado en todos los trabajos presentados en esta Tesis en los que se ha hecho uso de un analizador de triple cuadrupolo. Consiste en la monitorización de una transición concreta, es decir, en el primer cuadrupolo se aísla un ion de una m/z determinada que pasa a la celda de colisión. Allí se fragmenta en iones producto que llegan al tercer cuadrupolo, en el que se selecciona uno de ellos. Dado que durante todo el tiempo del análisis se está midiendo una o varias transiciones específicas, este modo de trabajo proporciona una elevada sensibilidad. Otra de las ventajas destacables del modo SRM es que la sensibilidad mejora debido a que, al ser menor el ruido de fondo, se consigue aumentar de manera significativa la relación S/N. Estos motivos convierten al triple cuadrupolo en modo SRM en una herramienta muy valiosa tanto para la cuantificación como para la confirmación de los compuestos.

En el analizador de tiempo de vuelo (TOF) los iones se separan en función del tiempo que tardan en atravesar un tubo de vuelo de longitud conocida. El tiempo empleado depende de la relación m/z de cada ión. El uso de reflectrones o espejos ópticos reenfoca sobre el detector los iones que tienen la misma m/z, consiguiendo de este modo una elevada resolución. Dicha resolución permite obtener medidas de masa exacta de los iones detectados.

En esta Tesis se ha hecho uso de un analizador híbrido QTOF, que combina dos analizadores distintos, un cuadrupolo y un analizador de tiempo de vuelo. La

posibilidad de obtener el espectro de iones producto con masa exacta permite identificar de manera inequívoca los compuestos presentes en una muestra.

El TOF ofrece la posibilidad de investigar la presencia de un compuesto tras haber realizado el análisis y adquirido los datos, es decir, realizar un análisis *a posteriori*. Esto es posible porque el espectro de masas que se adquiere en un análisis contiene información de toda la muestra, a diferencia de los métodos de QqQ donde sólo se obtiene información de los iones seleccionados antes de efectuar el análisis.

Las principales ventajas del QTOF, como son su elevada sensibilidad en modo de barrido de iones, su elevado poder de resolución, y la posibilidad de obtener medidas de masa exacta de los iones detectados, lo convierten en una herramienta de análisis ideal para el *screening* e identificación de compuestos orgánicos en el medio ambiente así como para la elucidación estructural. En cambio, este analizador posee un menor rango de respuesta lineal y menor sensibilidad que el QqQ, por lo que su aplicación en el campo cuantitativo es limitada.

En definitiva, el análisis mediante un triple cuadrupolo permite la cuantificación de los compuestos seleccionados con elevada sensibilidad y selectividad. En cambio, su poder de identificación y *screening* es limitado debido a su bajo poder de resolución (generalmente resolución unidad, es decir, diferencia entre masas que están separadas entre sí 1 uma) y a su baja sensibilidad en modo *full scan*. En este tipo de situaciones el TOF es una herramienta muy eficaz gracias a su elevado poder de resolución.

1.2.3 Análisis simultáneo de compuestos pertenecientes a diferentes grupos terapéuticos: métodos multiclase y multirresiduales

Los fármacos son un grupo de compuestos muy heterogéneo pues presentan diferentes estructuras y propiedades físico-químicas. Este hecho dificulta su análisis simultáneo.

Los primeros métodos desarrollados se centraron en la determinación de fármacos pertenecientes a la misma familia/grupo terapéutico. En cambio, en los últimos años la tendencia mayoritaria ha sido el desarrollo de métodos analíticos multiclase. Éstos permiten analizar simultáneamente compuestos pertenecientes a distintas familias químicas. Numerosos estudios publicados confirman la presencia de una gran variedad de fármacos pertenecientes a diferentes grupos terapéuticos.

La principal ventaja que ofrecen los métodos multiclase es que proporcionan una información mucho más amplia que los métodos centrados en el análisis de una única familia química. Asimismo, el tiempo de análisis y el coste que implicaría analizarlos mediante un método optimizado para cada grupo terapéutico es mucho menor. Sin embargo, debido a las distintas propiedades físico-químicas de los compuestos analizados, el desarrollo de un método multiclase resulta un proceso complicado. Obviamente, esta dificultad es mayor a medida que aumenta la multirresidualidad de los métodos. Así pues, se requiere encontrar un compromiso en la selección de las condiciones experimentales que permita el análisis simultáneo de todos ellos.

La etapa de tratamiento de la muestra (generalmente mediante extracción en fase sólida) suele presentar complicaciones ya que se deben encontrar unas condiciones genéricas que permitan la extracción simultánea de todos los compuestos. Variables como el tipo de sorbente utilizado, el pH de la muestra, el disolvente de elución empleado o el volumen han de evaluarse para conseguir una buena eficacia de extracción.

En cuanto a las condiciones cromatográficas, se deberían seleccionar las que aumenten la resolución, minimicen la coelución y permitan obtener una buena forma de pico para todos los compuestos analizados.

Otro de los aspectos que se ha de tener en cuenta a la hora de optimizar las condiciones cromatográficas de un método multiclase/multirresidual es la posibilidad de poder analizar todos los compuestos en una sola inyección. Así pues, un método de análisis "ideal" debería permitir la determinación simultánea de los compuestos ionizados en modo de ionización positivo y negativo. Para que sea posible se necesita, en primer lugar, un analizador capaz de cambiar de polaridad en un reducido intervalo de tiempo (por ejemplo, empleando tan solo 0.02 s como en el caso del analizador triple cuadrupolo utilizado en los trabajos presentados en esta Tesis). En segundo lugar, se debe seleccionar una fase móvil adecuada para el análisis de ambos tipos de compuestos.

También es necesario optimizar las condiciones de detección MS/MS. En este sentido es importante tener en cuenta que el número de transiciones que contiene un método tiene relación directa sobre la sensibilidad y la forma de pico. Generalmente, en los análisis mediante un triple cuadrupolo se suelen adquirir al menos dos transiciones SRM por compuesto. Cuando se adquieren muchas transiciones, como sucede en el caso de los métodos multirresiduales, éstas se dividen en diferentes ventanas en función del tiempo de retención. Se ha de tener en cuenta que se requieren al menos 10 puntos por pico para obtener picos cromatográficos bien definidos. Esto puede resultar problemático cuando se analiza un número elevado de compuestos porque generalmente las ventanas se solapan, de manera que cuantas más transiciones se midan simultáneamente, menos tiempo se empleará en monitorizar cada transición, provocando una disminución del número de puntos por pico. Esta situación puede conllevar una pérdida de sensibilidad y un empeoramiento de la forma de los picos cromatográficos. Los equipos más modernos, como los utilizados en esta Tesis, han conseguido mitigar esta problemática mediante el diseño de celdas de colisión que permiten trabajar a mayores velocidades de barrido, y en consecuencia, reducir el tiempo de monitorización de cada transición. Estas celdas están especialmente

diseñadas para trabajar con UHPLC donde debido a la estrechez de los picos cromatográficos se requiere un sistema de detección con altas velocidades de vaciado, capaces de trabajar con *dwell time* bajos. Recientemente algunas casas comerciales han desarrollado un software que simplifica este proceso. Así, de manera automatizada se seleccionan las condiciones de tiempo de monitorización más idóneas para cada transición en función del solapamiento entre las distintas funciones y de la anchura de pico.

Otro de los criterios que debe cumplir un método multiclase/multirresidual es que, para cada analito, los límites de detección y de cuantificación sean suficientemente bajos. Así, los métodos más recientes permiten medir bajos niveles de concentración, en el orden de ng/L o incluso por debajo, en distintos tipos de matrices.

Teniendo en cuenta todo lo expuesto en los párrafos anteriores, a medida que aumenta el número de compuestos de un método resulta más difícil encontrar las condiciones experimentales más idóneas para cada uno de los analitos. En ese caso se ha de llegar a una situación de compromiso que permita el análisis simultáneo de todos ellos aun sabiendo que, en ocasiones, los resultados obtenidos podrían haber sido mejores.

1.2.4 Efecto matriz

El efecto matriz es uno de los principales problemas de los métodos LC-MS/MS, especialmente para los que utilizan interfases ESI. La interfase APCI es menos susceptible a las interferencias de la matriz pero generalmente es menos sensible que la ESI. Además, como se ha comentado en el apartado 1.2.2, la interfase APCI resulta menos adecuada para los compuestos de mayor polaridad.

La IUPAC define el efecto matriz en química analítica como el efecto combinado de todos los componentes de la muestra distintos al analito sobre la medida del mismo. Si un componente específico puede identificarse como causante de un efecto, entonces se considera que es un interferente.

El efecto matriz se debe a la presencia de componentes presentes en la matriz que coeluyen con los analitos de interés. Aunque no se conoce el mecanismo exacto que lo origina, posiblemente tiene su origen en la competición entre el analito y los compuestos de la matriz que entran en la fuente de ionización al mismo tiempo. Allí ambos compiten por acceder a la superficie de las gotas cargadas y pasar a la fase gas, provocando una disminución (conocida como supresión de la señal) o un aumento (exaltación de la señal) de la eficiencia de la formación de los iones de los analitos (Taylor, 2005).

El grado de supresión o exaltación que sufre un analito en una muestra depende principalmente de la matriz en cuestión y también de las características físico-químicas de cada compuesto. Además, otros factores como el proceso de extracción, las condiciones cromatográficas, el tipo de instrumentación de espectrometría de masas utilizada, y las condiciones de ionización, influyen en el efecto matriz observado (Gosetti, 2010).

El efecto matriz puede afectar a la selectividad, reproducibilidad, exactitud, linealidad y límite de cuantificación del método (Gosetti, 2010). Las consecuencias del efecto matriz se convierten en errores en la cuantificación de los analitos ya que, si no se corrige, los valores de concentración de las muestras están sujetos a un error.

La corrección del efecto matriz en muestras de agua resulta complicada, especialmente en las matrices más complejas. Existen distintas estrategias dirigidas a eliminarlo o reducirlo en la medida de lo posible. Las más utilizadas en el análisis de muestras medioambientales son el uso de técnicas de extracción selectivas, la realización de una etapa de limpieza eficaz tras la extracción, la mejora de las condiciones de separación cromatográficas para evitar la coelución de los analitos con los interferentes, la realización de un calibrado en matriz, la dilución de la muestra y el uso de patrones internos.

Reducir el efecto matriz modificando el proceso de extracción de las muestras, es decir, aplicando procesos de extracción selectivos y/o aumentando la limpieza de las muestras tras la extracción, resulta complicado. El efecto matriz de las muestras que se

extraen mediante técnicas de extracción selectivas, tales como SPME o MIPs, es menor que cuando se utiliza SPE. Sin embargo, el uso de las técnicas selectivas no resulta adecuado para la extracción de todos los compuestos y por lo tanto, no pueden utilizarse para el análisis multiclase. Una explicación más detallada puede consultarse en el apartado 1.2.1.

Otra opción consistiría en incrementar el pretratamiento de muestra y la purificación. El principal inconveniente que presenta esta aproximación es que implica una mayor manipulación de las muestras y, en consecuencia, mayor probabilidad de que se produzcan errores analíticos. Además, implican un mayor tiempo de análisis (Fatta-Kassinos, 2011). También hay que tener en cuenta que cuando la extracción de las muestras se lleva a cabo mediante SPE, se preconcentran los analitos y también aquellos componentes de la matriz que son atrapados por el cartucho. En este caso la preconcentración SPE puede magnificar el efecto matriz, provocando el efecto opuesto al deseado (Gosetti, 2010).

La calibración en matriz es una de las estrategias más utilizadas en otros campos de análisis, como por ejemplo, en el análisis de alimentos. Consiste en realizar la cuantificación de los analitos mediante calibrados realizados en matriz de muestra blanco. De este modo tanto los analitos como los patrones utilizados sufren el mismo efecto matriz. Sin embargo, en el caso de muestras de agua su aplicación resulta difícil debido a la imposibilidad de encontrar muestras blanco con las que poder preparar el calibrado en matriz. Esto es especialmente complicado en el caso de muestras procedentes de EDAR. Además, la composición de la matriz de una muestra a otra varía, motivo por el que resulta casi imposible seleccionar una muestra blanco con un contenido de matriz similar al de las muestras a analizar.

El método de adiciones estándar consiste en añadir cantidades crecientes de analito a una cantidad fija de muestra. Se requiere un análisis previo de la muestra para estimar la concentración y poder realizar las adiciones correctas. En el caso de métodos multirresiduales, esta estimación se debería realizar para cada uno de los analitos considerados. Por ese motivo, su aplicación no resulta conveniente. Además, el método

de adiciones estándar puede conducir a errores cuando se analizan muestras a muy bajos niveles de concentración, como es el caso de los fármacos en el agua.

Otra de las posibilidades es la dilución de las muestras. Se trata de un procedimiento muy sencillo y eficaz con el que se consigue reducir la cantidad de matriz introducida dentro de la fuente de ionización. Al diluir la muestra con el mismo solvente con el que están preparados los patrones se minimiza el contenido de interferentes presentes en la matriz y las respuestas de las muestras se pueden llegar a hacer comparables con los patrones. El principal inconveniente de este método es que conlleva una disminución de la sensibilidad. El grado de dilución de las muestras depende de la combinación analito/matriz, es decir, de la sensibilidad de cada analito y del grado de exaltación/supresión. En el caso de matrices complejas (influente) se requiere una dilución elevada (por ejemplo, 1:10) mientras que en matrices con menor efecto matriz la aplicación de un factor de dilución de dos o tres suele ser suficiente para solucionar el problema.

El método de corrección más utilizado en el análisis de fármacos es, sin duda, el uso de patrones internos (Wong, 2009; Wille, 2012).

El procedimiento analítico habitual consiste en añadir una cantidad conocida de patrón interno al principio del proceso de extracción, ya que de este modo se corrigen los problemas producidos durante la extracción (posibles pérdidas) y el efecto matriz. Para que un patrón interno pueda corregir el efecto matriz su ionización debe verse afectada por los mismos interferentes y del mismo modo que el analito. Por ese motivo el patrón interno ideal es el mismo analito marcado isotópicamente.

Los patrones marcados con ^{13}C y D son los más utilizados ya que su abundancia en la naturaleza es extremadamente baja (alrededor del 1% y del 0.015%) y por lo tanto, no interfieren en la correcta cuantificación.

En el caso de los métodos multirresiduales, la adición de patrones internos está limitada ya que tanto desde el punto de vista económico como desde el punto de vista

de la disponibilidad de los compuestos marcados resulta imposible corregir cada compuesto con su propio compuesto marcado.

Cuando se dispone del compuesto marcado, la corrección se basa en que los iones de las analitos y sus análogos marcados correspondientes son químicamente equivalentes y la matriz les afecta de un modo similar (Castiglioni, 2006). Para que esto ocurra las estructuras químicas del analito y de su patrón interno deben ser similares. En caso contrario, el compuesto marcado isotópicamente podría presentar un comportamiento físico-químico diferente y, por consiguiente, conduciría a correcciones insatisfactorias. Este problema se ha observado al utilizar compuestos con un número elevado de isótopos deuterados (Stokvis, 2005; González-Antuña, 2010; Ripollés, 2012). Por ese motivo se aconseja que el número de átomos marcados oscile entre 2 y 5, aunque dependerá del analito en cuestión y de la posición que ocupen los átomos marcados.

En el caso de no disponer del propio analito marcado la alternativa más utilizada en el campo medioambiental consiste en la corrección con otro compuesto análogo. Su selección se basa en la similitud entre la estructura química del compuesto marcado y la del analito. Si no se dispone de uno semejante, la elección se hará en función del tiempo de retención de ambos, que ha de ser similar. Estas estrategias se abordarán en el Capítulo 2 de la presente Tesis.

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Capítulo 2

Determinación de fármacos en aguas mediante UHPLC-MS/MS con analizador de triple cuadrupolo

2.1 Introducción

2.1.1 Bibliografía

2.2 Determinación simultánea de los fármacos de mayor consumo, pertenecientes a diferentes grupos terapéuticos, en aguas residuales

2.2.1 Introducción

2.2.2 Artículo científico 1: *Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra-high-pressure liquid chromatography-tandem mass spectrometry*

Journal of Chromatography A, 1217 (2010) 622-632

2.2.3 Discusión de los resultados (artículo científico 1)

2.2.4 Bibliografía

2.3 Determinación simultánea de fármacos, incluyendo numerosos antibióticos, en aguas medioambientales y residuales. Estudio y corrección del efecto matriz en el proceso de cuantificación

2.3.1 Introducción

2.3.2 Artículo científico 2: *Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry*

Journal of Chromatography A, 1218 (2011) 2264-2275

2.3.3 Discusión de los resultados (artículo científico 2)

2.3.4 Bibliografía

2.1 Introducción

Como se ha comentado en el Capítulo 1, los analizadores de triple cuadrupolo trabajando en modo SRM son una herramienta muy poderosa para el análisis cuantitativo. Su elevada sensibilidad en dicho modo de trabajo posibilita la determinación de diferentes contaminantes orgánicos en aguas a muy bajos niveles de concentración, entre ellos, los fármacos.

Esta configuración de trabajo, donde los analitos se seleccionan antes de realizar la inyección en el sistema LC-MS, se podría denominar *pre-target analysis*. En el campo de fármacos, esta metodología permite determinar simultáneamente un número notable de compuestos, incluyendo desde unos pocos analitos hasta varias decenas (Kaspryzk-Hordern, 2009; Ferrer, 2010; Gros, 2012). Sin embargo, su principal inconveniente es la falta de capacidad para detectar otros compuestos presentes en una muestra que no hayan sido seleccionados previamente, aunque estos se encuentren a elevados niveles de concentración.

El modo *pre-target* implica la optimización de las condiciones MS/MS de cada compuesto mediante la infusión de su patrón analítico en disolución. En primer lugar, se debe establecer el modo de ionización y el voltaje de cono para definir el ion precursor. A continuación, se aísla el ion precursor y se determina la energía de colisión óptima para obtener los iones producto característicos. Entre ellos, se seleccionan aquellos que se quieren monitorizar, en función de la sensibilidad (abundancia del ion) pero también teniendo en cuenta el criterio de la selectividad de la transición, ya que si ésta es poco selectiva cabe el riesgo de reportar falsos positivos. Por ese motivo, se debe

evitar seleccionar transiciones derivadas de pérdidas comunes como agua, monóxido de carbono, ácido fórmico...

En principio, la adquisición de dos transiciones debería ser suficiente para identificar y confirmar la presencia de un compuesto. Este es el criterio de confirmación que se sigue en la mayoría de los métodos desarrollados para el análisis de fármacos en muestras medioambientales, tomando como base la Decisión 2002/657/EC de la Comisión Europea. Se trata, en realidad, de unas pautas propuestas para la cuantificación y confirmación de contaminantes y residuos orgánicos en muestras de alimentos de origen animal. Sin embargo, debido a la ausencia de directrices en el campo medioambiental, en los últimos años se ha aplicado también en este tipo de análisis.

Esta Decisión distingue dos grupos de contaminantes. El grupo A incluye a aquellos compuestos que están prohibidos, y el grupo B a las sustancias para las que se ha establecido un límite máximo de residuo permitido (MRL). Este último es el caso de los fármacos de uso veterinario.

Dicha Decisión establece un sistema de puntos de identificación (IPs) para poder asegurar la presencia de un compuesto en una muestra. El número de IPs depende de la técnica utilizada, diferenciando entre MS y MS^n, y entre instrumentos de baja resolución y de alta resolución. Así, para instrumentos de baja resolución en modo MS/MS, como es el caso de un analizador de triple cuadrupolo, se requieren al menos 4 IPs (un ion precursor y dos iones producto), es decir, dos transiciones por analito para una identificación fiable.

En la Tabla 2.1 se muestran los valores de IPs asociados a cada técnica:

Tabla 2.1 Relación entre las distintas técnicas MS y sus IPs asociados

Técnica MS	IPs obtenidos por cada ion
Espectrometría de masas de baja resolución (LR)	1.0
LR-MS^n ion precursor	1.0
LR-MS^n ion producto	1.5
Espectrometría de masas de alta resolución (HR)	2.0
HR-MS^n ion precursor	2.0
HR-MS^n ion producto	2.5

Además, para poder identificar correctamente un compuesto en una muestra se ha de cumplir la relación de intensidad (*ion ratio*) entre las transiciones seleccionadas. Esta relación debe coincidir con la obtenida para un patrón, de acuerdo con unas tolerancias establecidas en función de su intensidad relativa (Tabla 2.2). Asimismo, el tiempo de retención entre el compuesto y el patrón no debe desviarse más del 2.5%.

Tabla 2.2 Tolerancias máximas permitidas para la confirmación de contaminantes
Decisión 2002/657/EC de la Comisión Europea

Relación de intensidades (Q/q)	Desviación permitida LC-MS LC MS^n
1 - 2	± 20%
2 - 5	± 25%
5 - 10	± 30%
> 10	± 50%

En todos los trabajos presentados en esta Tesis se ha hecho uso de la técnica de separación conocida como cromatografía líquida de ultra resolución, cuyas siglas en inglés son UHPLC (*ultra-high performance (or pressure) liquid chromatography*). A pesar de que se trata de una técnica ya consolidada, no existe un consenso en la

comunidad científica sobre el uso del término *performance* o *pressure*, aunque el primer término parece ser el escogido por la mayoría de los autores.

Esta técnica de separación se basa en el uso de columnas rellenas de partículas con un tamaño inferior a 2 μm. Su uso proporciona una mayor resolución cromatográfica y un aumento de la sensibilidad mediante la obtención de picos cromatográficos más estrechos (generalmente, 5-10 segundos de anchura) y más definidos, gracias a la menor difusión de las moléculas de analito a través de la fase estacionaria. Además, se reduce considerablemente el tiempo de análisis respecto a la cromatografía convencional (HPLC).

Para poder aprovechar las ventajas que ofrecen las columnas de UHPLC se requieren velocidades lineales altas, es decir, flujos de fase móvil elevados. Esto genera elevadas presiones en el sistema por lo que se precisa de una instrumentación avanzada capaz de trabajar a altas presiones. Obviamente, las partículas de las columnas también han de soportar estas presiones de trabajo.

La primera partícula compatible que se creó fue una partícula híbrida que combina las propiedades de los rellenos inorgánico (sílice) y orgánico (polimérico), de 1.7 μm, conocida con las siglas BEH (*Ethylene Bridged Hybrid*). Estas columnas poseen un enlace trifuncional que les confiere una elevada estabilidad y un sangrado de columna muy bajo. Pueden incorporar distintos ligandos, aunque lo más habitual es el uso de una fase estacionaria enlazada de cadenas hidrocarbonadas C18 que se unen a la sílice mediante puentes de etileno. Posteriormente, se desarrolló una partícula de sílice de alta resistencia (HSS, *High Strenght Silica*) de 1.8 μm, con cadenas C18 enlazadas. Estas columnas proporcionan una mayor retención de los analitos en comparación con las BEH. Ambos tipos de columnas se han utilizado en los trabajos que se presentan en este capítulo. La primera compañía que desarrolló este tipo de tecnología fue Waters Corporation pero en la actualidad otras ofrecen productos similares.

El empleo de la UHPLC también requiere de sistemas de detección basados en espectrometría de masas con altas velocidades de adquisición, capaces de trabajar con tiempos de monitorización (*dwell times*) bajos. Para ello, se ha modificado la celda de

colisión de los sistemas de detección, permitiendo la reducción del tiempo de residencia de un ion en su interior y por tanto, el tiempo de monitorización empleado respecto a los sistemas convencionales HPLC. De este modo, estos analizadores se ajustan a la mayor rapidez cromatográfica de UHPLC pero manteniendo al menos diez puntos por pico cromatográfico. Además, al reducir el *dwell time* es posible adquirir un mayor número de transiciones SRM por compuesto simultáneamente, facilitando de esta manera la confirmación de los analitos.

En los apartados siguientes se presentan cuatro trabajos en los que se ha hecho uso del acoplamiento UHPLC-MS/MS con analizador de triple cuadrupolo para la determinación cuantitativa de fármacos. En el primero de ellos se desarrolla un método para la determinación de los 20 fármacos más consumidos en España. Esta lista de compuestos se amplió posteriormente mediante la incorporación de aproximadamente 30 antibióticos, dando como resultado el método presentado en el segundo trabajo. En el tercer trabajo se presentan datos de concentración de los fármacos seleccionados en muestras de influente y de efluente procedentes de distintas EDAR situadas en la provincia de Castellón y se evalúa la eficiencia del proceso de depuración en cuanto a su posible eliminación. Por último, se desarrolla un método para el análisis combinado de fármacos seleccionados y de una serie de productos de cuidado personal.

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Water Research, 43: 363-380

2.2 Determinación simultánea de los fármacos de mayor consumo, pertenecientes a diferentes grupos terapéuticos, en aguas residuales

2.2.1 Introducción

En el presente trabajo se muestra la optimización, validación y aplicación del primer método analítico desarrollado en esta Tesis para la determinación de fármacos en aguas superficiales y residuales.

La selección de los compuestos se realizó en base a datos oficiales proporcionados por el Ministerio de Sanidad Español. Dicho organismo publica cada año los grupos de fármacos más consumidos atendiendo a la clasificación Anatómica, Terapéutica y Química, más conocida como clasificación ATC (*Anatomic, Therapeutic, Chemical*), y los principios activos de mayor consumo en el Sistema Nacional de Salud a través de recetas médicas.

El documento ordena los principios activos por importe total a precio de venta al público. Además, también proporciona información sobre el número de envases totales (en miles de unidades) vendidos de cada subgrupo ATC así como de los principios activos más consumidos.

En este trabajo se seleccionaron inicialmente los diez fármacos con mayor número de envases consumidos y los diez de mayor importe. En muchos casos los compuestos más consumidos (en número de envases) se correspondían con los de mayor importe total, por ejemplo, la atorvastatina y el omeprazol. Tras una amplia revisión bibliográfica decidimos incluir otros fármacos que, aunque no estaban catalogados dentro de las listas de los diez más consumidos, se habían detectado en las aguas en estudios previos publicados. Este es el caso de los antiinflamatorios diclofenaco, ketoprofeno y naproxeno, o de los reguladores lipídicos gemfibrozil y bezafibrato. Cabe mencionar que muchos de estos compuestos pueden adquirirse sin receta médica. Por ello resulta comprensible su elevada frecuencia de detección en el medio ambiente.

La selección de los compuestos en base a los criterios señalados nos permitió tener una visión realista de la presencia de los fármacos en el medio ambiente, a diferencia de otros métodos publicados que aparentemente no tienen en cuenta los contaminantes que realmente pueden estar presentes en las muestras. En muchos

casos no se justifican los criterios de selección de los compuestos y da la impresión de que estos se han seleccionado atendiendo a criterios muy diversos tales como la disponibilidad comercial de los patrones, la elección de compuestos con características físico-químicas similares con el fin de realizar un único tratamiento de muestra, y la capacidad para poder analizarlos en un solo método analítico.

En algunos trabajos publicados el criterio de selección de los compuestos se realiza en base a las listas de sustancias prioritarias en el agua establecidas por distintos organismos (Directiva Europea 2000/60/CE, lista EPA CCL-3). Sin embargo, estas listas incluyen principalmente plaguicidas y metales y sólo desde hace poco tiempo contienen algunos fármacos. La EPA en el año 2009 actualizó su lista de contaminantes prioritarios en agua potable y únicamente se incluyó un fármaco, en concreto un antibiótico (eritromicina). Recientemente se ha hecho pública una propuesta de modificación de la Directiva Europea 2000/60/CE y, por primera vez, se ha incluido un fármaco, concretamente el antiinflamatorio diclofenaco.

En el método desarrollado en el presente apartado, analitos de distinta naturaleza (ácidos, neutros y básicos) se preconcentran empleando un único tratamiento de muestra y posteriormente se analizan en una sola inyección. Este último aspecto supone una ventaja con respecto a un gran número de métodos multirresiduales publicados. Muchas veces los compuestos que se ionizan en modo positivo y en negativo se determinan en dos análisis diferentes (Spongberg, 2008; Batt, 2008; Gros, 2009; Ferrer, 2010), lo que implica la utilización de distintas fases móviles y, en ocasiones, de diferentes columnas cromatográficas. En consecuencia, el tiempo de análisis y el coste son más elevados que en los métodos analíticos en los que todos los compuestos se analizan en un único análisis, como es el caso del método que aquí se presenta.

En cuanto al tratamiento de muestra empleado en este trabajo, cabe decir que satisface la tendencia actual de los métodos multirresiduales en los que mediante un único tratamiento de muestra se preconcentran todos los analitos. Como se ha comentado en el Capítulo 1, este proceso entraña una cierta dificultad por la diversidad

de propiedades físico-químicas de los compuestos (pK_a, polaridad, solubilidad, estabilidad, etc.), por lo que muchas veces resulta complicado obtener recuperaciones satisfactorias para todos ellos utilizando un único método de extracción. Algunos investigadores han optado por utilizar distintos cartuchos que presentan una alta selectividad para compuestos ácidos o básicos (Zuccato, 2010) en lugar de una única extracción.

Otro de los aspectos destacables de este estudio es el uso de la técnica de separación UHPLC acoplada a un analizador de triple cuadrupolo en tándem (UHPLC-MS/MS). Hoy en día se trata de una técnica muy utilizada, pero en el año 2010 (año de publicación del artículo que se presenta a continuación) tan sólo se habían publicado unos pocos artículos haciendo uso de la cromatografía UHPLC para la determinación de fármacos en muestras de agua.

Por último, la adquisición de tres transiciones por compuesto, en lugar de las dos que habitualmente se utilizan, nos permitió identificar con gran fiabilidad los compuestos detectados en las muestras analizadas, especialmente en algunos casos conflictivos que se muestran en el artículo. Cabe recordar que esto fue posible gracias a la combinación de la UHPLC con un analizador con elevada velocidad de barrido.

2.2.2 Artículo científico 1

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Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry

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ABSTRACT

In this work, an ultra high-pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method has been developed for the simultaneous quantification and confirmation of the 20 most consumed pharmaceuticals in Spain in urban wastewater and surface water samples. The scope of the method included acidic, neutral and basic compounds belonging to different therapeutic classes and allows their simultaneous determination in just a single injection, giving realistic information of the most widely consumed pharmaceuticals in only one analysis. An enrichment step based on solid-phase extraction using Oasis HLB cartridges was carried out, followed by UHPLC-MS/MS measurement with a fast-acquisition triple quadrupole mass analyzer. It allowed working with short dwell times and made possible to acquire three simultaneous SRM transitions per compound to assure a reliable identification. Several isotope-labelled internal standards were used as surrogates to correct SPE losses, as well as matrix effects that notably affect quantification of analytes. The method was validated in surface water and effluent and influent urban wastewater at different concentrations from 0.005 μg/L (surface water) to 1.25 μg/L (influent wastewater). The optimized method was applied to the analysis of 84 urban wastewater samples (influent and effluent), with the result that 17 out of 20 compounds monitored were detected in the samples. Analgesics and anti-inflamatories, cholesterol lowering statin drugs and lipid regulators were the major groups found, with diclofenac, ketoprofen, naproxen, 4-aminoantipyrine, bezafibrate, gemfibrozil and venlafaxine being the most frequently detected. The highest concentration level reached was 277 μg/L for salicylic acid in influent wastewater.

1. Introduction

Investigation of pharmaceuticals in the environment has become an important issue in the last years due to their large worldwide consumption and to their potential adverse effects on the animal and human health. Previous studies have demonstrated that pharmaceuticals are continuously being released in the environment, mainly through excreta, disposal of unused or expired products, or from pharmaceuticals discharges. Most of them are not completely removed from wastewater treatment plants (WWTPs), and can enter in ground and drinking water at low concentrations, from ng/L to μg/L [1,2]. Nowadays, not reliable data are available about long-term effects in the environment yet, and there is a need of performing ecotoxicological studies to know their concentration levels and to evaluate the possible toxic effects associated to their exposition. Therefore, the development of sensitive, selective and wide-scope methods is of major importance to have realistic data on their presence in both surface and wastewater.

As most pharmaceuticals are polar compounds, the technique of choice is, at the moment, HPLC coupled to mass spectrometry (MS), preferably to tandem MS. The development of faster and more sensitive methods is nowadays feasible using techniques like ultra high-pressure liquid chromatography (UHPLC), which has become one of the most suitable analytical tools for the determination of contaminants in environmental samples [3,4]. This technology provides greater resolution, increased sensitivity and high speed of analysis. The use of UHPLC in combination to tandem MS using fast analyzers makes possible working with short dwell times, thus increasing the number of selected reaction monitoring (SRM) transitions acquired simultaneously per compound. This increases confidence in the identification of analytes detected in samples.

UHPLC-MS/MS is increasingly being used for the determination of different organic contaminants in water. Typically, an off line preconcentration step is required to reach the sensitivity necessary to

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Keywords

Pharmaceuticals; Ultra high pressure liquid chromatography; Tandem mass spectrometry; Triple quadrupole; Multi-class analysis; Surface and urban wastewater; Matrix effects.

1. Introduction

Investigation of pharmaceuticals in the environment has become an important issue in the last years due to their large worldwide consumption and to their potential adverse effects on the animal and human health. Previous studies have demonstrated that pharmaceuticals are continuously being released in the environment, mainly through excreta, disposal of unused or expired products, or from pharmaceuticals discharges. Most of them are not completely removed from wastewater treatment plants (WWTPs), and can enter in ground and drinking water at low concentrations, from ng/L to µg/L [1, 2]. Nowadays, not reliable data are available about long-term effects in the environment yet, and there is a need of performing ecotoxicological studies to know their concentration levels and to evaluate the possible toxic effects associated to their exposition. Therefore, the development of sensitive, selective and wide-scope methods is of major importance to have realistic data on their presence in both surface and wastewater.

As most pharmaceuticals are polar compounds, the technique of choice is, at the moment, HPLC coupled to mass spectrometry (MS), preferably to tandem MS. The development of faster and more sensitive methods is nowadays feasible using techniques like ultra high-pressure liquid chromatography (UHPLC), which has become one of the most suitable analytical tools for the determination of contaminants in environmental samples [3, 4]. This technology provides greater resolution, increased sensitivity and high speed of analysis. The use of UHPLC in combination to tandem MS using fast analyzers makes possible working with short dwell times, thus increasing the number of selected reaction monitoring (SRM) transitions acquired simultaneously per compound. This increases confidence in the identification of analytes detected in samples.

UHPLC-MS/MS is increasingly being used for the determination of different organic contaminants in water. Typically, an off line pre-concentration step is required to reach the

sensitivity necessary to detect the low concentrations normally present in samples, in the range of ng/L [5 - 9].

Until now, most of attention has been paid to the presence of antibiotics in water [10-14]. In the last few years, methods developed for pharmaceuticals tend to simultaneously determine compounds belonging to different therapeutical groups, in contrast to previous methods that were focused on one specific group [15-17]. Thus, multi-class methods provide a more realistic knowledge about the presence of pharmaceuticals in water. As pointed out by some authors, in many published methods target analytes were selected because they could be included in a single method, due to their similar charge or ionization mode, or because reference standards or isotope-labelled internal standards were commercially available, or because these compounds had been previously detected [7]. However, to have a realistic view of the presence of pharmaceuticals in the environment, the most relevant compounds from the consumption point of view should be selected. In the present work, we have compiled information about the most consumed pharmaceuticals in Spain with medical prescription in the last years [18]. All these compounds were included in the method developed, together with some other pharmaceuticals that had been previously detected in surface water and urban wastewater by other authors [2, 15-17, 19].

A drawback when developing multi-residue multi-class methods comes from the quite different physico-chemical characteristics of the analytes, which makes difficult to find the most suitable chromatographic and MS conditions for all compounds; then a satisfactory compromise should be reached for the simultaneous analysis of all of them. However, regarding ionization mode, it is hard to find in the scientific literature applications analyzing positive and negative ionized pharmaceuticals in a single injection. Typically, positive and negative ionized analytes are determined in separate analysis, even using different column and mobile phases [7, 20-21]. This is also problematic in the solid-phase extraction (SPE) step, because the extraction efficiency is compound dependent, and is affected by several variables such as the type of the sorbent used, sample pH, polarity of the solvent used for elution, or elution volume. Another key point is that selected chromatographic and MS conditions must be satisfactory for all type of water samples analyzed. This aspect is problematic when dealing with complex matrices, like urban wastewater samples, because co-eluting substances may lead to undesirable signal suppression/enhancement effects. In order to solve matrix effects, the use of isotope-labelled internal standards (ISs) seems to be the preferred strategy. This approach has been widely applied in the field of pharmaceuticals analysis [1, 2, 6, 7, 20, 21].

The aim of this work is to develop rapid, selective and sensitive analytical methodology based on simultaneous sample enrichment by off-line SPE followed by UHPLC-MS/MS for the simultaneous determination of 20 acidic, neutral and basic pharmaceuticals widely consumed in Spain. All compounds, both measured under electrospray positive and negative ionization mode, are determined simultaneously in just one injection and acquiring three SRM transitions per compound. This allows their simultaneous detection, quantification and confirmation, making possible to reach more than 4 identification points (IPs) [22, 23]. The most sensitive transition is used for quantification, while the other two allow the safe confirmation of the identity of the compounds detected in samples. The suitability of using several isotope-labelled ISs was evaluated to compensate matrix effects in surface water, but especially in wastewater where severe matrix effects were observed. The developed method was applied to the analysis of 84 wastewater samples from three WWTPs located at the Castellón province (Spain).

2. Experimental

2.1. Reagents and chemicals

The pharmaceuticals analyzed were selected accordingly to the following criteria: (i) the most consumed active principles with medical prescription in Spain [18] (ii) previous information reported in scientific literature about occurrences in surface and wastewater.

Acetaminophen (paracetamol), salicylic acid, ibuprofen, 4-aminoantipyrine, omeprazole, ketoprofen, naproxen, bezafibrate, diclofenac, gemfibrozil, pravastatin sodium and enalapril maleate salt were purchased from Sigma–Aldrich (Steinheim, Germany). Lorazepam, alprazolam, venlafaxine hydrochloride, risperidone and paroxetine hydrochloride were from LGC Promochem (London, UK). Atorvastatin and olanzapine were from Toronto Research Chemicals (Ontario, Canada). Pantoprazole was obtained by dissolving Anagastra® powder in HPLC-grade water. Isotopically labelled compounds were omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, salicylic acid-d_3 and ibuprofen-d_3 from CDN Isotopes (Quebec, Canada) and atorvastatin-d_5, paroxetine hydrochloride-d_4 and olanzapine-d_3 from Toronto Research Chemicals (Toronto, Canada). HPLC-grade methanol and HPLC-grade acetonitrile were purchased from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q Gradient A10 (Millepore, Bedford, MA, USA). Formic acid (HCOOH, content >98%) and ammonium acetate (NH_4Ac, reagent grade) were supplied by Scharlau (Barcelona, Spain).

Stock standard solutions were prepared dissolving 25 mg, accurately weighted, in 50 mL methanol, obtaining a final concentration of 500 mg/L. For LC-MS analysis, the individual stock solutions were mixed and diluted with methanol to give a final concentration of around 1 mg/L and subsequently diluted, when required, with HPLC-grade water to obtain working mixed solutions of pharmaceuticals. These working solutions were used for spiking samples in the validation study and also for preparation of calibration standards, which were prepared in methanol–water (10:90, v/v).

Individual stock solutions of isotope-labelled IS were also prepared in methanol. A mixed working solution at 100 μg/L (for IS ionizing in positive mode) and at 1 mg/L (for IS ionizing in negative mode), was prepared in water and used as surrogate.

Due to the low stability of some compounds, mainly omeprazole, working solutions of pharmaceuticals were renewed monthly.

SPE cartridges used were Oasis HLB (60 mg), Oasis HLB (200 mg) and Oasis MCX (150 mg) from Waters (Milford, MA, USA).

2.2. Liquid chromatography

UHPLC analysis was carried out using an Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was carried out with an Acquity UPLC BEH column, 1.7 μm, 50 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 60 °C and the sample manager was maintained at 5 °C. Mobile phase consisted of water/methanol gradient both 0.1 mM NH_4Ac and 0.01% HCOOH. The methanol percentage changed linearly as follows: 0 min, 5%; 1.5 min, 5%; 2 min, 30%; 3 min; 50%; 5 min, 70%; 6 min, 90%; 7 min, 90%; 7.1 min, 5%. Analysis run time was 9 min. Mobile phases were filtered under vacuum through 0.22 nylon membrane filters.

2.3. Mass spectrometry

For UHPLC analysis, a TQD (quadrupole–hexapole–quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters Corp., Milford, MA, USA) was used.

Drying gas, as well as nebulising gas, was nitrogen generated from pressurized air in a N_2 LC-MS (Claind, Teknokroma, Barcelona, Spain). Cone gas and desolvation gas flows were set at 60 L/h flow and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a pressure of 2×10^{-3} mbar in the T-Wave cell. Capillary voltages of −3.0 and 3.5 kV were used in negative and positive ionization mode, respectively. Interface temperature and source temperature were optimized at 500 and 120 °C, respectively. Dwell times of 0.01 s/scan were selected.

Masslynx NT (Microsmass, Manchester, UK) software was used to process quantitative data.

2.4. Recommended procedure

All influent samples (IWW) as well as those effluent (EWW) and surface water samples (SW) with observable suspended particulate matter were centrifuged at 4500 rpm for 5 min before loading the SPE cartridges.

Oasis HLB (60 mg) cartridges were previously conditioned with 3 mL of methanol and 3 mL of HPLC-grade water. 100 mL of water sample were spiked with the mix IS working solution to give a final concentration of 0.1 μg/L for those isotope-labelled IS determined in positive mode (omeprazole-d_3, acetaminophen-d_4, atorvastatin-d_5, paroxetine-d_4, olanzapine-d_3) and of 1 μg/L for those determined in negative mode (salicylic acid-d_3, diclofenac-d_4, ibuprofen-d_3). Then, the sample was passed through the cartridge by gravity (flow rate around 3 mL/min). After drying under vacuum, analytes were eluted with 5 mL of methanol. The extract was evaporated to dryness under a gentle nitrogen stream (40 °C) and finally reconstituted with 1 mL methanol–water (10:90, v/v). Analyses were performed by injecting 20 μL of the final extract in the UHPLC-MS/MS system. Experimental MS conditions are given in Table 1. Quantification of samples was performed using calibration standards in solvent (methanol–water 10:90), which also contained the labelled IS. Thus, relative areas were used for quantification purposes.

Table 1
MS/MS optimized conditions for selected compounds.

Compound	Therapeutic group	LOD (pg)	MW	Q Transition	Cone (V)	C.E.(eV)	q_1 Transition	C.E.(eV)	q_2 Transition	C.E.(eV)	Q/q_1	Q/q_2
Acetaminophen	Analgesic and anti-inflamatories	1.7	151.1	152.1 > 110.1	30	15	152.1 > 93.0	25	152.1 > 65.0	30	5.5	9.1
4-Aminoantipyrine (metabolite of metamizol)		0.5	203.3	204.2 > 56.0	30	20	204.2 > 83.0	15	204.2 > 94.0	20	4.9	8.1
Diclofenac		8.2	295.0	294.1 > 250.1	30	10	296.1 > 252.1	30	-	-	1.0	-
Ibuprofen		52.4	206.1	205.2 > 161.1	30	10	-	-	-	-	-	-
Ketoprofen		6.4	254.1	253.2 > 209.2	20	5	-	-	-	-	-	-
Naproxen		7.6	230.1	185.2 > 170.1	30	10	229.2 >185.2*	5	229.2 > 170.1*	20	2.9	4.5
Salicylic acid		17.0	138.0	137.1 > 93.0	30	15	137.1 > 65.0	25	-	-	21.2	-
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	0.3	558.3	559.4 > 440.3	45	20	559.4 > 250.2	45	559.4 > 276.2	40	1.3	3.3
Pravastatin		12.4	424.2	423.4 > 321.2	40	15	423.4 > 101.1	30	423.4 > 303.2	20	1.0	2.1
Bezafibrate		1.6	361.1	360.2 > 274.1	30	15	362.2 >276.2	20	360.2 > 154.0	30	4.1	1.8
Gemfibrozil		12.8	250.2	249.3 > 121.0	30	15	249.3 > 127.0	10	-	-	16.1	-
Paroxetine	Antidepressants	2.9	329.1	330.3 > 70.1	50	20	330.3 >44.1	30	330.3 > 192.1	20	1.6	2.3
Venlafaxine		0.3	277.2	278.3 > 58.0	30	15	278.3 > 260.3	15	260.3 > 58.0**	15	2.1	3.3
Omeprazole	Anti-ulcer agents	1.3	345.1	346.3 > 198.1	30	10	346.3 > 136.1	35	346.3 > 151.1	20	1.3	1.9
Pantoprazole		4.4	383.1	384.2 > 200.1	25	35	384.2 > 138.1	10	384.2 > 153.1	15	1.0	1.6
Olanzapine	Psychiatric drugs	0.5	312.1	313.3 > 256.2	45	25	313.3 > 84.1	25	313.3 > 198.1	35	1.3	10.9
Risperidone		0.7	410.2	411.3 > 191.2	50	30	411.3 > 82.1	60	411.3 > 110.1	50	8.1	8.7
Alprazolam	Ansiolitics	0.3	308.1	309.2 > 281.2	60	25	309.2 > 205.2	40	309.2 > 274.2	25	0.9	3.6
Lorazepam		0.5	321.2	321.2 > 275.1	40	20	323.2 > 277.1	20	321.2 > 303.2	15	1.3	4.4
Enalapril	Cardiovasculars	0.2	376.2	377.4 > 234.2	35	20	377.4 > 91.1	55	377.4 > 160.2	30	1.5	3.1

Abbreviations: MW (monoisotopic molecular weigh), Q (quantification), q (confirmation), C.E. (collision energy).
* Cone Voltage: 20 V
** Cone Voltage: 40 V

2.5. Validation study

Method accuracy (expressed as recovery percentage) and precision (expressed as repeatability in terms of relative standard deviation (RSD)) were evaluated by recovery experiments of target compounds in surface water (SW), effluent wastewater (EWW) and influent wastewater (IWW), spiked at different concentration levels (0.005, 0.025 and 0.05 μg/L in SW; 0.1 and 0.5 μg/L in EWW; 0.25 and 1.25 μg/L in IWW). Experiments were performed by quintuplicate (n = 5) for each type of water sample tested and for each spiking level. Recoveries between 70 and 120% with RSD lower than 20% were considered as satisfactory.

The limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio of 10 from SRM chromatograms of samples spiked at the lowest validation level tested, from the quantification transition. In those particular cases where a sample blank was not feasible (several analytes were normally present in all effluent and influent wastewaters), the LOQ was estimated from the "blank" chromatograms without spiking the sample. In this case, the analyte concentration for the peak observed in the "blank" sample was quantified. Then, the LOQ was estimated for S/N = 10 taking into account the analyte concentration found in the "blank". The instrumental limit of detection (LOD) was estimated for S/N = 3 from the chromatograms of standards at the lowest concentration level tested in the calibration curve.

The linearity of the method was studied by analyzing standard solutions in triplicate at concentrations typically ranging from 0.25 to 500 μg/L, although final concentrations tested depended on the sensitivity reached for each analyte. Satisfactory linearity using weighed (1/X) least squares regression was assumed when the correlation coefficient (r) was higher than 0.99, based on analyte peak areas measurement, and when residuals were lower than 30% without significant trend.

2.6. Application to real samples

A total number of 84 urban wastewater samples (42 IWW and 42 EWW) were collected in polyethylene high-density bottles and stored at <−18 °C until analysis. Before analysis, samples were thawed at room temperature. Samples consisted on 24-h composite urban wastewater samples, and were collected from three WWTPs of the Castellón province (Benicàssim, Burriana and Castellón de la Plana). Samples were collected along one complete week in two different months (June 2008 and January 2009).

3. Results and discussion

3.1. MS and MS/MS optimization

Full-scan and MS/MS mass spectra were obtained from infusion of 1 mg/L methanol/water (50:50, v/v) individual standard solutions at a flow rate of 10 μL/min. The multi-class characteristics of selected pharmaceuticals made that 12 out of 20 compounds presented positive ionization meanwhile the rest were determined under negative mode. Acetaminophen and ibuprofen were ionized in both negative and positive modes. For the first compound, positive ionization was selected, while negative ionization mode was used for ibuprofen because of the better sensitivity reached under these modes.

All compounds showed an abundant $[M+H]^+$ or $[M-H]^-$ ion. These were selected as precursor ions, except for naproxen that showed better sensitivity when using an in-source fragment as precursor ion by increasing the cone voltage. For venlafaxine an additional sensitive transition was also obtained under in-source fragmentation (see Table 1).

For diclofenac, bezafibrate and lorazepam the presence of one chlorine atom in their structure allowed using two different precursor ions (corresponding to ^{35}Cl and ^{37}Cl isotopes, respectively).

In this work, a fast-acquisition triple quadrupole analyzer has been used. It allows reducing dwell times and increasing the number of SRM transitions acquired simultaneously. Dwell times as low as of 0.01 s could be used without resolution and/or sensitivity losses. This made feasible to acquire three simultaneous SRM transitions for each compound to assure a reliable identification. 4 out of 20 analytes showed poor fragmentation (ibuprofen, ketoprofen, salicylic acid and diclofenac). For these specific compounds, only one or two transitions could be monitored.

In order to acquire at least 10 points per peak and to ensure that enough time was spent on each transition to avoid data loss, SRM transitions where divided into seven overlapping elution-time windows (four elution windows for compounds determined under positive ionization mode and three windows for those under negative mode). It is worth to mention that the low positive-to-negative-switching time (0.02 s) of the tandem mass instrument used in our work allows this favourable overlapping between positive and negative time windows.

Mass spectrometry parameters selected, precursor and product ions, as well as instrumental LODs are shown in Table 1.

3.2. Chromatographic optimization

In order to optimize the chromatographic separation, different mobile phases (methanol and acetonitrile) with different additives (HCOOH and NH_4Ac at various concentrations) were tested. A short UPLC BEH column (50 mm × 2.1 mm, 1.7 μm) was chosen. It allowed performing an efficient chromatographic separation for all the 20 analytes in only 7 min.

For those compounds determined under positive ionization, sensitivity improved when NH_4Ac was added, both in water and in methanol mobile-phase solvents. For those compounds determined in negative ionization mode, the use of mobile phases without any additive provided better ionization yield, but it resulted in a non-desirable peak shape. This problem was solved by adding NH_4Ac, which allowed improving the poor chromatographic behaviour of these compounds.

The addition of formic acid (0.01% HCOOH) improved the chromatographic separation (reduction of peak tailing and better resolution) of several compounds measured in positive mode. It also favoured the retention of the negatively charged compounds in the LC column. Therefore, methanol and water, both containing 0.1 mM NH_4Ac and 0.01% HCOOH, were finally chosen as mobile phases for the simultaneous chromatographic separation of both positive and negative ionized analytes.

Enalapril exhibited two poor resolved peaks because it is present as a mixture of cis- and trans-conformers around the amide bond [21, 24]. To obtain a single peak for quantitative analysis of enalapril, the column temperature was increased from 40 to 60 °C without affecting the chromatographic separation of the other compounds.

3.3. Solid Phase Extraction

A detailed study was carried out on the most relevant parameters – type of sorbent, pH of the sample and elution conditions – that affect the recovery of target compounds.

First of all, the extraction efficiency of two cartridges was tested using HPLC-grade water spiked with the analytes. Cartridges used were Oasis HLB (200 mg) and Oasis MCX (150 mg). Oasis HLB was tested at four pH values (8.5, 7, 4.5 and 2) while Oasis MCX, a mixed polymeric-cation exchange sorbent, was tested at pH 2, by acidifying the water sample with HCOOH. As methanol seems to be an efficient solvent for the elution of polar contaminants from different SPE cartridges, it was chosen for elution when evaluating the SPE process [25]. The vast majority of compounds determined in negative mode showed satisfactory recoveries using both cartridges, except for salicylic acid, which was partially lost during the SPE process. However, the best recoveries for pharmaceuticals determined in positive mode were obtained with Oasis HLB. Therefore, HLB cartridge was chosen for subsequent experiments. The performance of the sorbents tested at different pHs for all analytes is summarized in Figure 1.

Regarding the effect of sample pH, satisfactory data were obtained using Oasis HLB cartridges at pH 7 for most compounds, although olanzapine, 4-aminoantipyrine and atorvastatin were poorly recovered. A slight improvement was observed for 4-aminoantipyrine and atorvastatin at pH 8.5, but this pH affected negatively to the recoveries of omeprazole and pantoprazole.

The difficulty for extraction of atorvastatin, the pharmaceutical most commonly used for the treatment of hypercholesterolemia, has been related to its instability, due to a possible interconversion of the lactone and acidic form [26, 27]. Therefore, pH is one of the most important variables to minimize this interconversion. In this work, the potential problems associated to this analyte were solved by using its own isotope-labelled IS, obtaining satisfactory recoveries in all matrices tested.

As the objective of this work was to simultaneously extract the 20 selected acidic, neutral and basic pharmaceuticals, with quite different physico-chemical characteristics, SPE at pH 7 with Oasis HLB was selected as a compromise.

In order to determine if low recoveries for olanzapine, 4-aminoantipyrine and atorvastatin were consequence of exceeding the breakthrough volume, different volumes (10, 25, 50 and 100 mL) of spiked water were passed through the HLB cartridges at pH 7. Similar results were obtained in all cases; therefore, the volume of water samples was maintained at 100 mL.

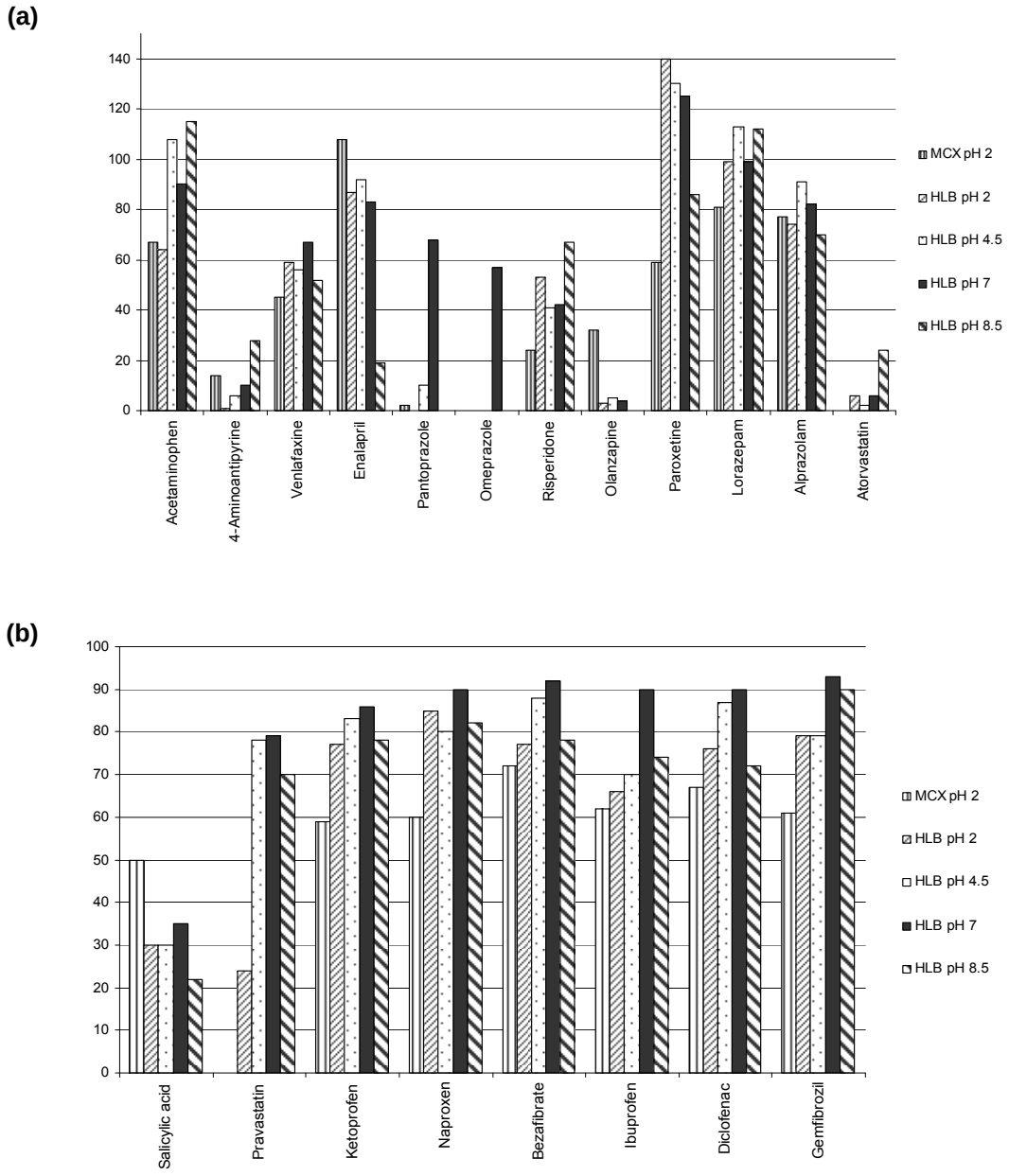

Figure 1. Recoveries obtained after extraction of selected analytes with Oasis HLB (200 mg) and Oasis MCX (150 mg) cartridges at different sample pH values. (a) Compounds analyzed in positive mode and (b) compounds analyzed in negative mode.

Once the type of cartridge and sample pH was selected, three elution solvents (methanol, acetone and acetonitrile) were evaluated. Using acetone, enalapril and paroxetine were partially recovered. Methanol and acetonitrile did not show relevant differences although recoveries were slightly better when eluting with methanol; so, this solvent was selected for elution (5 mL).

A comparison between Oasis HLB 60 and 200 mg was carried out, eluting with 5 and 10 mL methanol, respectively. Similar results were obtained for all compounds, except for atorvastatin and risperidone, which recoveries were slightly improved using HLB 60 mg cartridge. A possible explanation might be that these analytes were less retained in 60 mg cartridges, and subsequently they might be more easily eluted. Finally, Oasis HLB 60 mg cartridges were selected. This allowed reducing solvent volume and the time necessary to evaporate the extract.

The slightly high recovery for paroxetine (around 130%) might be due to unknown compounds released from the SPE cartridges that would coelute with this analyte producing a slightly signal enhancement [28], as the blank performed with HPLC-grade water did not show any interferent peak. In any case, the use of paroxetine isotope labelled as surrogate IS allowed us to obtain satisfactory recoveries when the method was applied to spiked real-world samples (see next section).

3.4. Method validation

Analytical characteristics of the method were evaluated in three types of water samples (SW, EWW and IWW) that were spiked at different concentrations.

Linearity was studied in the range 0.25–500 μg/L for all selected compounds. Depending on the sensitivity reached for each analyte different linear responses were obtained: (1) alprazolam, lorazepam, enalapril, omeprazole, atorvastatin, venlafaxine, risperidone and 4-aminoantipyrine showed satisfactory linearity along this range; (2) acetaminophen, olanzapine, pantoprazole, diclofenac, bezafibrate and gemfibrozil showed linear response from 1 to 500 μg/L; (3) the rest of compounds showed good results in the range 5–500 μg/L. In all these cases, residuals were below 30% and correlation coefficients by linear or quadratic curves (risperidone) were greater than 0.99.

Accuracy and precision were estimated from recovery experiments of target analytes at different concentration levels. Recoveries were determined by comparing the concentrations obtained in spiked samples after applying the recommended procedure, using calibration curves with standards in solvent. Several isotope-labelled ISs were added as surrogates in order to compensate compounds' losses during the SPE process and/or matrix effects. In the case of EWW and IWW, it was not feasible to get a true blank, as all samples analyzed contained one or more analytes included in this work. So, EEW and IWW were previously analyzed and the concentrations found for target compounds present in the "blank" samples were subtracted from the spiked samples. It must be taken into account that subtracting the analyte amount present in the "blank" sample normally leads to higher errors in the recovery and RSD calculation.

The method was tested at three fortification levels in SW. As Table 2 shows, the 0.025 μg/L level could not be validated for four analytes determined in negative mode due to their lower sensitivity, but all of them were determined satisfactorily at 0.05 μg/L (recovery from 70 to 120%). The high sensitivity typically observed for analytes determined in positive mode allowed us validating satisfactorily the method at a level as low as 0.005 μg/L. All compounds, which isotope-labelled IS was available, were quantified using its corresponding analyte-labelled IS. The rest of selected compounds, as matrix effects in surface water tested were not much relevant, could be quantified without using IS. Only 4-aminoantipyrine presented low recoveries at all fortification levels due to SPE pre-concentration losses that could not be properly corrected.

As can be seen in Table 3, recoveries and precision in effluent wastewater were satisfactory at 0.1 μg/L for 16 compounds out of 20 tested. 4-Aminoantipyrine and gemfibrozil were not validated at this level due to high concentration found in the "blank" sample (around 8–10 times higher than the spiking level). Salicylic acid and ibuprofen could not be validated at the lowest level assayed due to the lower sensitivity observed for these compounds.

Table 2
Method validation for surface water (SW). Recovery (%) and relative standard deviation (RSD. %) for five replicates.

Compound	Polarity (ES)	t_R (min)	0.005 µg/L		0.025 µg/L		0.05 µg/L		LOQ (ng/L)	I.S. Used
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
Acetaminophen	+	1.60	[a]	-	115	3	107	3	9.3	Acetaminophen-d_4
Olanzapine	+	2.66	[a]	-	63	11	68	11	5.7	Olanzapine-d_3
4-Aminoantipyrine	+	2.76	55	5	48	1	27	1	0.8	
Salicylic acid	-	3.13	[a]	-	[a]		99	15	44	Salicylic acid-d_4
Risperidone	+	3.18	82	9	76	11	88	6	2.0	
Venlafaxine	+	3.30	70	8	70	4	110	2	0.2	
Enalapril	+	3.76	84	5	90	3	97	6	3.3	
Omeprazole	+	3.80	83	8	77	9	85	7	3.2	Omeprazole-d_3
Pantoprazole	+	3.83	[a]	-	87	15	88	13	20	
Paroxetine	+	3.76	[a]	-	94	9	97	9	19	Paroxetine-d_4
Lorazepam	+	4.39	93	19	86	2	96	2	4.3	
Alprazolam	+	4.42	82	8	78	4	83	4	2.9	
Pravastatin	-	4.40	[a]	-	[a]	-	90	22	25	
Ketoprofen	-	4.57	[a]	-	[a]	-	83	5	23	
Naproxen	-	4.69	[a]	-	76	12	103	6	21	
Bezafibrate	-	4.81	[a]	-	95	10	99	10	7.6	
Atorvastatin	+	5.52	101	13	100	7	106	4	0.8	Atorvastatin-d_5
Diclofenac	-	5.58	[a]	-	117	12	90	7	11	Diclofenac-d_4
Ibuprofen	-	5.75	[a]	-	[a]		106	12	39	Ibuprofen-d_3
Gemfibrozil	-	6.16	[a]	-	80	14	93	5	12	

Abbreviations: ES (Electrospray ionization).t_R (retention time).

[a] Not estimated due to the low sensitivity at the fortification level tested.

As expected, method validation in influent wastewater was the most complicated case, especially at the lowest fortification level. Because of the impossibility to obtain true blanks, several samples were previously analyzed and that sample containing the lowest pharmaceutical concentration levels was selected for validation. Due to their high complexity and elevated organic matter content, it was necessary to dilute five times the IWW samples before validation. Spiking levels tested were 0.25 and 1.25 μg/L in the non-diluted raw sample (i.e. 0.05 and 0.25 μg/L in the 5-fold diluted sample). In general, recoveries and precision were satisfactory for most compounds at both fortification levels. 4-Aminoantipyrine, naproxen, ibuprofen and atorvastatin could not be validated at the low level due to high concentrations found in the "blank". As can be seen in Table 4, at 1.25 μg/L concentration level, a few compounds (salicylic acid, risperidone, ibuprofen, lorazepam and atorvastatin) showed recoveries around 120%, but precision was satisfactory in all cases (RSD < 10%).

The high complexity of sample matrix in wastewater samples (both EWW and IWW), affected considerably the recovery values of many compounds. Thus, in this type of samples, the use of IS to correct matrix effects was compulsory. This led to recoveries mostly within the desired range of 70–120%. Each compound was corrected with its labelled IS, when available, and the others were corrected with ISs eluting at closer retention times. For example, gemfibrozil and pravastatin were corrected using an analogue IS (diclofenac-d_4), obtaining satisfactory recoveries. Alprazolam, lorazepam, risperidone and enalapril could be quantified without using IS correction with acceptable recoveries and precision (see Table 3).

The method presented satisfactory precision for all type of water samples with most RSD values below 15%. LOQs were estimated for the three water samples tested. LOQs ranged from 0.2 to 25 ng/L for SW, from 3.6 to 85 ng/L for EWW and from 8.6 to 200 ng/L for IWW. The two exceptions were salicylic acid and ibuprofen, as the sensitivity was lower for these compounds, with the result of higher LOQs (see Table 2, Table 3 and Table 4). Concerning instrumental LODs, they ranged from 0.2 to 13 pg.

Table 3
Method validation for effluent wastewater (EWW). Recovery (%) and relative standard deviation (RSD. %) for five replicates.

Compound	Polarity (ES)	t_R (min)	0.1 µg/L		0.5 µg/L		LOQ (ng/L)	I.S. Used
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
Acetaminophen	+	1.60	114	4	103	2	85	Acetaminophen-d_4
Olanzapine	+	2.66	66	9	70	5	11	Olanzapine-d_3
4-Aminoantipyrine	+	2.76	[a]	-	79	2	44[b]	Acetaminophen-d_4
Salicylic acid	-	3.13	[c]	-	104	9	427	Salicylic acid-d_4
Risperidone	+	3.18	114	9	91	4	5.9	
Venlafaxine	+	3.30	88	12	91	4	3.6	Omeprazole-d_3
Enalapril	+	3.76	80	1	87	1	6.6	
Omeprazole	+	3.80	88	13	79	6	11	Omeprazole-d_3
Pantoprazole	+	3.83	103	18	89	3	33[b]	Omeprazole-d_3
Paroxetine	+	3.76	92	3	91	5	43	Paroxetine-d_4
Lorazepam	+	4.39	85	2	87	2	30[b]	
Alprazolam	+	4.42	75	4	78	3	11	
Pravastatin	-	4.40	96	13	70	3	22	Diclofenac-d_4
Ketoprofen	-	4.57	87	12	84	15	72[b]	Diclofenac-d_4
Naproxen	-	4.69	91	10	84	8	30[b]	Diclofenac-d_4
Bezafibrate	-	4.81	86	13	86	4	9.5[b]	Diclofenac-d_4
Atorvastatin	+	5.52	106	4	99	2	7.4	Atorvastatin-d_5
Diclofenac	-	5.58	83	8	84	2	53[b]	Diclofenac-d_4
Ibuprofen	-	5.75	[c]	-	120	15	247	Ibuprofen-d_3
Gemfibrozil	-	6.16	[a]	-	102	17	18[b]	Diclofenac-d_4

Abbreviations: ES (Electrospray ionization). t_R (retention time).

[a] Not estimated due to the high analyte levels found in the "blank" sample (around 0.8 µg/L for 4-aminoantipyrine and 0.9 µg/L for gemfibrozil).

[b] LOQ determined from the "blank" sample chromatogram (non-spiked).

[c] Not estimated due to the low sensitivity at the fortification level tested.

Table 4
Method validation for effluent wastewater (EWW). Recovery (%) and relative standard deviation (RSD. %) for five replicates.

Compound	Polarity (ES)	t_R (min)	0.25 µg/L		1.25 µg/L		LOQ (ng/L)	I.S. Used
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
Acetaminophen	+	1.60	127	8	105	3	112[a]	Acetaminophen-d_4
Olanzapine	+	2.66	66	7	68	8	13	Olanzapine-d_3
4-Aminoantipyrine	+	2.76	[b]	-	113	5	31[a]	Acetaminophen-d_4
Salicylic acid	-	3.13	[c]	-	123	5	974	Salicylic acid-d_4
Risperidone	+	3.18	86	6	120	5	8.6	
Venlafaxine	+	3.30	78	7	93	5	10	Omeprazole-d_3
Enalapril	+	3.76	112	14	95	6	23	
Omeprazole	+	3.80	102	7	102	2	29	Omeprazole-d_3
Pantoprazole	+	3.83	121	24	100	8	66	Omeprazole-d_3
Paroxetine	+	3.76	68	17	89	6	196	Paroxetine-d_4
Lorazepam	+	4.39	120	8	117	2	54	
Alprazolam	+	4.42	97	5	91	3	33	
Pravastatin	-	4.40	[c]	-	99	8	118	Diclofenac-d_4
Ketoprofen	-	4.57	55	8	71	4	109[a]	Diclofenac-d_4
Naproxen	-	4.69	[b]	-	100	6	49[a]	Diclofenac-d_4
Bezafibrate	-	4.81	113	8	98	7	20[a]	Diclofenac-d_4
Atorvastatin	+	5.52	[b]	-	116	1	31[a]	Atorvastatin-d_5
Diclofenac	-	5.58	112	13	98	9	137[a]	Diclofenac-d_4
Ibuprofen	-	5.75	[b]	-	124	6	642[a]	Ibuprofen-d_3
Gemfibrozil	-	6.16	118	6	96	6	48[a]	Diclofenac-d_4

Abbreviations: ES (Electrospray ionization). t_R (retention time).

[a] LOQ determined from the "blank" sample chromatogram (non-spiked).

[b] Not estimated due to the high analyte levels found in the "blank" sample (around 1.7 µg/L for 4-aminoantipyrine, 1.0 µg/L for naproxen, 0.5 µg/L for atorvastatin and 6.9 µg/L for ibuprofen).

[b] LOQ determined from the "blank" sample chromatogram (non-spiked).

[c] Not estimated due to the low sensitivity at the fortification level tested.

3.5. Application to environmental water samples

The method developed in this paper was applied to the analysis of 84 urban wastewater samples (42 influents and 42 effluents) (see Table 5).

Table 5
Summary of the results obtained in the monitoring of pharmaceuticals in influent and effluent wastewater from three urban WWTP of the Castellón province (total numberof samples analyzed 84).

Compound	Therapeutic group	Influent wastewater (n = 42)		Effluent wastewater (n = 42)	
		% positive findings	Maximum level (µg/L)	% positive findings	Maximum level (µg/L)
Acetaminophen	Analgesic and anti-inflamatories	100	201.3[a]	0	n.d.
4-Aminoantipyrine (metabolite of metamizol)		100	6.45	100	1.68
Diclofenac		100	1.49	100	0.74
Ibuprofen		98	39.8a	33	<LOQ
Ketoprofen		100	1.17	100	0.62
Naproxen		100	3.58	100	0.72
Salicylic acid		76	276.7[a]	26	236.1[a]
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	100	0.45	76	0.16
Pravastatin		26	0.24	30	0.17
Bezafibrate		100	0.46	100	0.39
Gemfibrozil		100	2.12	100	1.24
Paroxetine	Antidepressants	0	n.d.	0	n.d.
Venlafaxine		100	0.52	100	0.30
Omeprazole	Anti-ulcer agents	0	n.d.	43	0.10
Pantoprazole		0	n.d.	65	0.18
Olanzapine	Psychiatric drugs	0	n.d.	0	n.d.
Risperidone		0	n.d.	0	n.d.
Alprazolam	Ansiolitics	0	n.d.	38	<LOQ
Lorazepam		0	n.d.	55	0.06
Enalapril	Cardiovasculars	96	0.29	0	n.d.

[a] Samples were previously diluted to fit to the linearity range of the method.

In every sequence of analysis, the calibration curve was injected twice, at the beginning and at the end. Two quality control samples (QCs), i.e. a “blank” water sample (previously analyzed) fortified at the two validated levels, were also analyzed for quality control. QC recoveries were considered satisfactory if they were in the range 70–120% for every analyte.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and the two confirmation (q_1 and q_2) transitions, and comparing them with ion-ratios from a reference standard. The finding was considered positive when experimental ion-ratios were within the tolerance range [22, 23]. In spite that two SRM transitions per compound are normally considered sufficient for a reliable confirmation of the compound identity, in this work we acquired three transitions in order to increase the confidence

of the confirmation process. This is in the line of our previous work, where we have described some drawbacks when using two transitions [29].

It is interesting to mention the advantages of acquiring three SRM transitions per analyte. Fig. 2 shows the UHPLC-MS/MS chromatograms for bezafibrate reference standard (Figure 2a) and for an effluent wastewater sample that might had been reported as negative for bezafibrate if only the q_1 transition had been acquired (Figure 2b). The reason for doubting about this positive (or negative) sample was that the ion-ratio was out of the tolerance range. However, the second confirmation transition (q_2) was in agreement with the reference standard indicating that the sample was positive to bezafibrate actually. It seemed that q_1 transition was interfered by a co-eluting isobaric compound sharing one product ion (*m*/*z* 276) with the analyte. Under these circumstances, the acquisition of the third transition allowed us to confirm the presence of bezafibrate in the sample.

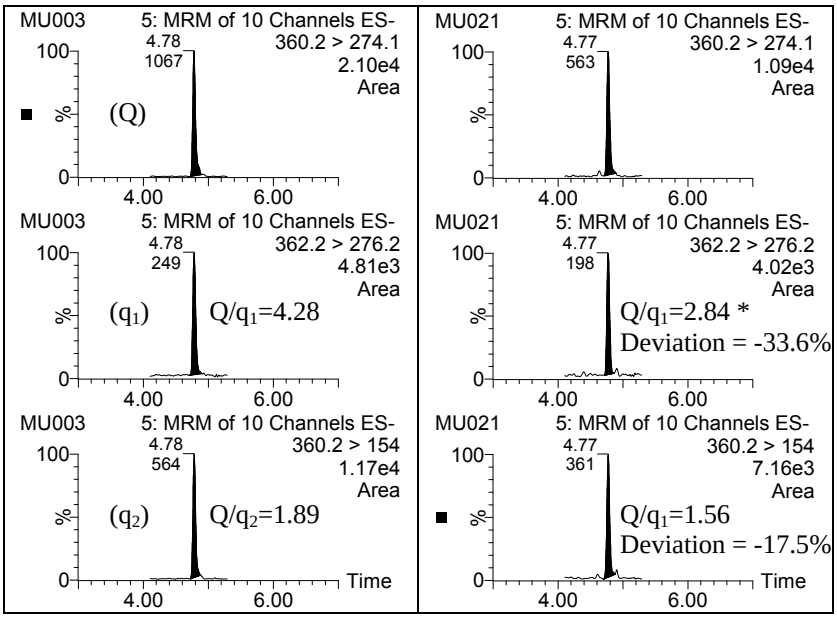

Figure 2. Selected UHPLC-MS/MS chromatograms for (a) bezafibrate reference standard, (5 µg/L) and (b) effluent wastewater sample (0.05 µg/L of bezafibrate). Quantification transition (*Q*), confirmation transitions (q_1 and q_2). * Ion-ratio deviation out of tolerance.

Analytes were quantified as described in the previous sections. However, in only a few EWW and IWW samples, QCs recoveries for venlafaxine were not satisfactory using omeprazole-d_3 as IS (>150%). This fact is accordance to other studies published about the use of analogues IS [8, 30-31]. Only using the own analyte-labelled IS assures a satisfactory correction in all types of samples, because the use of analogues IS does not always assures an efficient matrix effects correction.

Analgesics and anti-inflamatories, cholesterol lowering statin drugs and lipid regulators were the major groups detected in urban wastewater. The highest concentrations corresponded to acetaminophen, salicylic acid and ibuprofen in IWW. In relation to analgesics and anti-inflamatories, acetaminophen was found in all IWW analyzed, and 84% of IWW samples had to be diluted and re-analyzed to fit the linearity range of the method. However, this compound was not detected in effluent wastewater. Diclofenac, naproxen, ketoprofen and 4-aminoantipyrine were present in all IWW and EWW samples analyzed at concentration levels normally in the range of high ng/L or low µg/L. Among them, the highest concentrations corresponded to 4-aminoantipyrine with average concentrations of 2.78 µg/L in IWW and 0.89 µg/L in EWW.

Concerning lipid regulators, gemfibrozil and bezafibrate were present in all influent and effluent samples analyzed. The highest concentrations were found for gemfibrozil in IWW, with an average value of 1.38 µg/L, while in EWW the average level was 0.57 µg/L. Atorvastatin, the most consumed statin pharmaceutical, was also present in most of IWW and EWW, although its levels were lower than for lipid regulators.

A general overview to EWW pharmaceuticals data show that salicylic acid was by far the compound present at highest levels. Diclofenac, naproxen, ketoprofen and 4-aminoantipyrine and other compounds like venlafaxine, lorazepam and pantoprazole were frequently detected in EWW, although normally at concentrations below 0.5 µg/L.

Three of the compounds selected in this work were not detected in neither IWW nor EWW: the antidepressant paroxetine and the psychiatric drugs olanzapine and risperidone. It seems that searching for metabolites of these three compounds is necessary to follow their impact on aquatic environment.

When analyzing water samples with high matrix load, like urban influent wastewater, chromatographic retention time shifts may occur. Under this situation, the acquisition of additional confirmatory SRM transitions could help for analyte confirmation. As an example,

Figure 3 shows an influent wastewater sample positive for venlafaxine. The analyte retention time differed notably between the standard (3.30 min) and the sample (3.72 min). However, the ion-ratio when using the q_1 transition was within tolerance range. This apparent contradiction was solved by acquiring a second confirmation transition (q_2) that allowed us to assure that the sample was positive for venlafaxine, as the second ion-ratio also was in agreement with the reference standard.

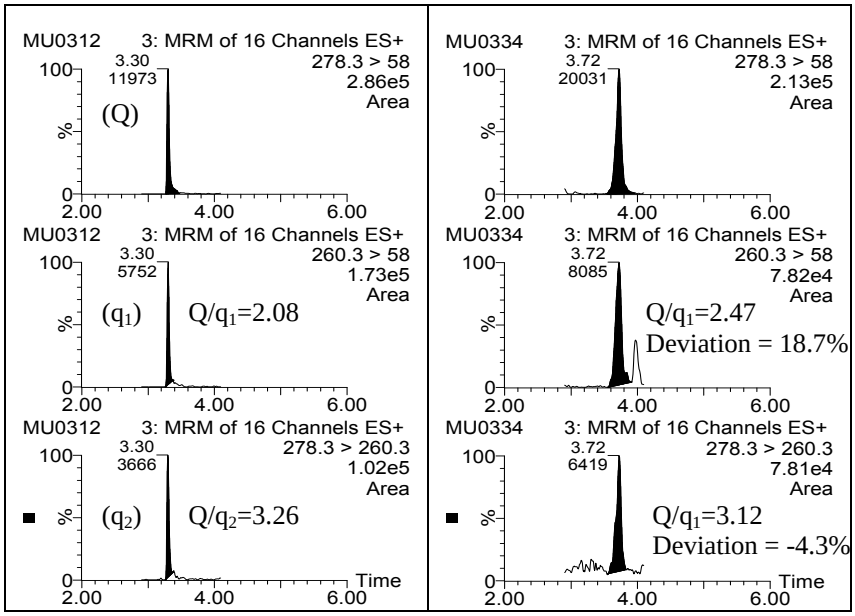

Figure 3. Selected UHPLC-MS/MS chromatograms for (a) venlafaxine reference standard, (5 µg/L) and (b) influent wastewater sample, (0.3 µg/L of venlafaxine). Quantification transition (*Q*), confirmation transitions (q_1 and q_2).

This fact was observed for other analytes as well, especially in IWW. Figure 4 shows another illustrative example, where chromatograms of the reference standard (Figure 4a) and the influent QC sample (Figure 4b) presented different retention times for risperidone.

In all influent wastewater samples, strong signal suppression was observed for olanzapine and its IS (olanzapine-d_3). The IS signal even disappeared in some samples, notably decreasing sensitivity for this compound in IWW.

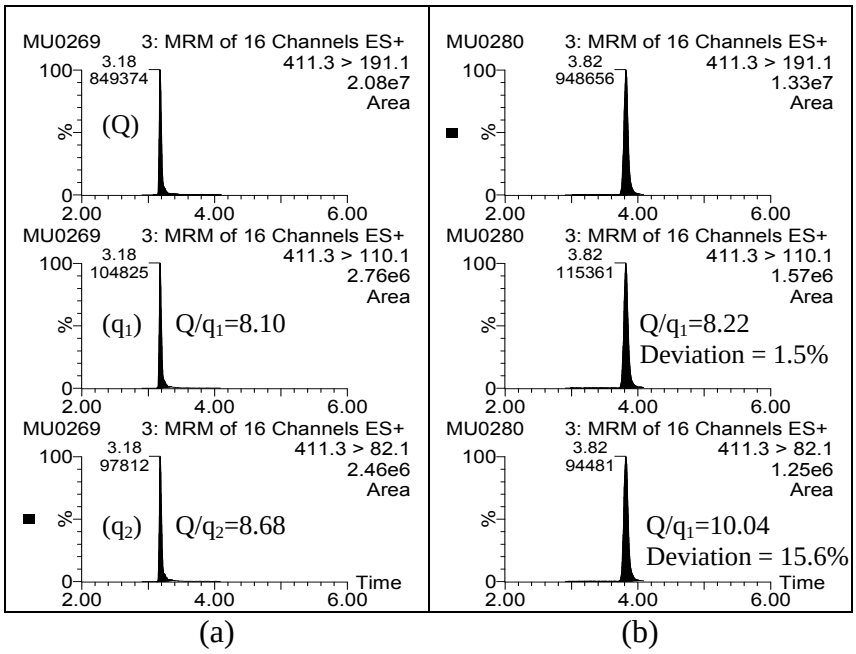

Figure 4. Selected UHPLC-MS/MS chromatograms for (a) risperidone reference standard and (b) influent wastewater spiked at the highest level validated. Quantification transition (*Q*), confirmation transitions (q_1 and q_2).

In the light of our preliminary data, WWTPs seemed to have good removal efficiency for some compounds, e.g. acetaminophen and enalapril. For the rest of pharmaceuticals, although their concentrations were lower than in IWW, positive samples were still found in EWW, and in some particular cases pharmaceuticals concentrations were slightly higher in the effluent.

As an illustrative example, Fig. 5 shows the UHPLC-MS/MS chromatograms for an effluent water sample (only quantitative transition is shown). As can be seen, the sample was positive for 12 out of 20 target compounds. However, none of the pharmaceuticals detected exceeded 1 µg/L.

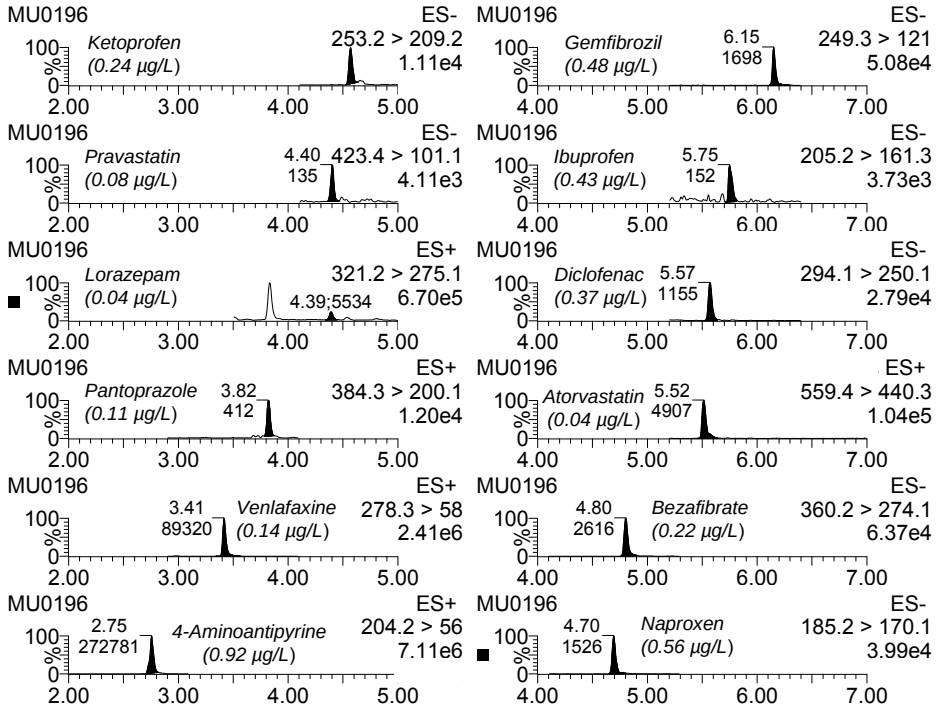

Figure 5. UHPLC-MS/MS chromatograms (quantification transition) for an effluent wastewater sample (Burriana, January 2009).

4. Conclusions

Rapid, selective and sensitive analytical methodology, based on the use of UHPLC-MS/MS with triple quadrupole analyzer has been developed for the simultaneous multi-class determination of 20 acidic, neutral and basic pharmaceuticals in urban wastewater and surface water. Target analytes were selected among the most widely consumed in Spain and corresponded to the therapeutic groups of analgesic and anti-inflamatories, cholesterol lowering statin drugs and lipid regulators, antidepressants, anti-ulcer agents, psychiatric drugs, ansiolitics and cardiovasculars. The method allows the simultaneous extraction of all selected analytes, with quite different physico-chemical characteristics, in a single step using Oasis HLB cartridges at neutral pH. The use of fast-acquisition triple quadrupole analyzer makes feasible selecting short dwell times (0.01 s) and acquiring up to three simultaneous SRM transitions per compound to assure a reliable identification for all analytes. Thanks to the use of both, UHPLC

and this MS analyzer, the safe quantification and identification of all analytes is feasible at very low concentration levels with a chromatographic run of only 9 min. Thus, it is not necessary to perform two analyses, for positive and negatively ionized compounds, as all of them can be determined in only one injection using an optimized mobile phase for positive and negative analytes. The method has been validated in three types of water at different concentrations depending on the sensitivity reached for every analyte/matrix combination. Illustrative of the excellent sensitivity of the method is that a level as low as 5 ng/L could be validated in surface water, still detecting the three SRM transitions acquired per analyte. Recoveries for most selected compounds were higher than 70% with very few exceptions. In urban wastewater the use of labelled internal standards has allowed a satisfactory correction of matrix effects that suffered most pharmaceuticals, mainly in influent wastewater.

The developed method has been applied to monitor pharmaceuticals in influent and effluent wastewater 24-h composite samples collected at two different seasons, showing a widespread occurrence of pharmaceuticals. The advantages of acquiring three SRM transitions per analyte for a reliable identification have been illustrated in several cases. The third transition helped us to confirm the presence of bezafibrate in EWW, where the ion-ratio of the other transition was out of tolerance limits. In influent wastewater a notable shift in chromatographic retention times was observed in positive samples. Again, the use of three SRM transitions reinforced reliability in the analyte confirmation process.

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2.2.3 Discusión de los resultados (artículo científico 1)

Optimización de las condiciones MS/MS

En primer lugar, se optimizaron los parámetros de masas para los iones precursores y producto de todos los analitos seleccionados. Los compuestos se ionizaron en modo positivo a excepción de ocho compuestos que se determinaron en modo negativo. En todos los casos se seleccionó como ion precursor $[M+H]^+$ o $[M-H]^-$ excepto para un compuesto (naproxeno) para el que su facilidad de fragmentación a bajas energías nos obligó a seleccionar su ion producto más abundante como ion precursor, promoviendo su fragmentación en el cono mediante la aplicación de un voltaje de cono elevado. En el caso de la venlafaxina, obtuvimos una transición adicional mediante fragmentación en el cono. En aquellos compuestos con un átomo de cloro en su estructura fue posible escoger entre dos iones precursores distintos: ^{35}Cl (abundancia isotópica 2/3) y ^{37}Cl (1/3). Se escogieron las transiciones más abundantes con el fin de evitar diferencias notables entre la transición de cuantificación y las de confirmación y facilitar de este modo la identificación del compuesto a bajas concentraciones.

Aunque la adquisición de dos transiciones por compuesto se considera suficiente para confirmar su presencia, en este estudio decidimos adquirir tres transiciones para aumentar más aún la confianza del proceso de confirmación. Para cuatro analitos (ibuprofeno, ketoprofeno, ácido salicílico y diclofenaco) solamente fue posible seleccionar una o dos transiciones debido a la escasa presencia de iones producto.

Optimización cromatográfica y del proceso de extracción

Para seleccionar la fase móvil se probaron varios disolventes (metanol y acetonitrilo) con distintos aditivos (HCOOH y NH_4Ac), a varias concentraciones. Con el fin de analizar simultáneamente todos los analitos, es decir, tanto los que se ionizan en positivo como los que lo hacen en modo negativo, se alcanzó una situación de

compromiso que consistió en el uso de ambos aditivos en agua y metanol. En concreto, NH4Ac (0.1 mM) mejoraba la sensibilidad de los compuestos determinados en modo positivo, mientras que HCOOH (0.01%) mejoraba la separación cromatográfica de éstos y favorecía la retención en la columna de los compuestos ionizados en modo negativo.

El enalapril en disolución se encuentra como una mezcla de dos isómeros conformacionales (cis/trans). Con el fin de obtener un solo pico cromatográfico se decidió aumentar la temperatura de la columna cromatográfica (60° C) (Figura 2.1). Este efecto se atribuye a la aceleración de los cambios conformacionales entre los dos isómeros a elevadas temperaturas, dando lugar a un único pico.

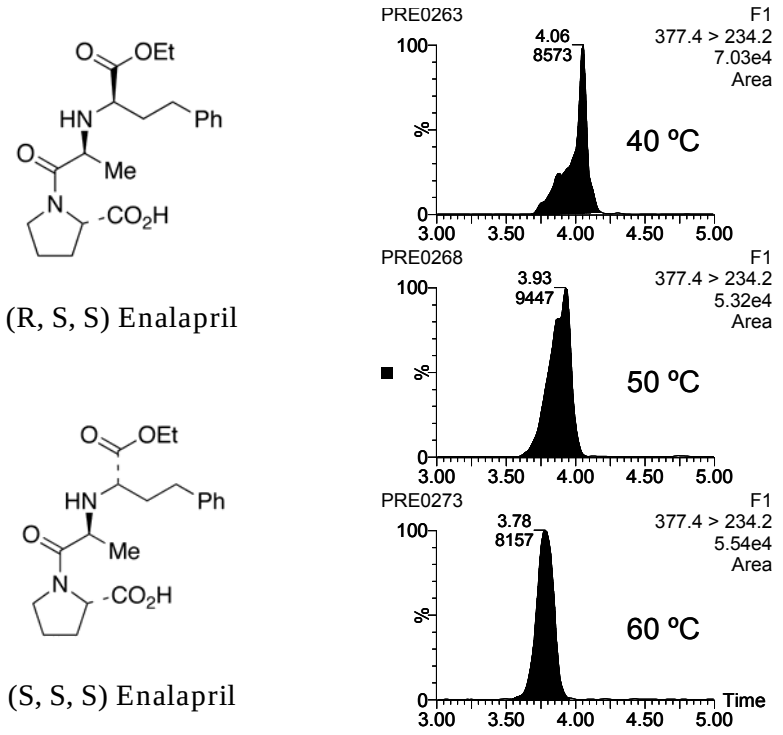


Figura 2.1 Estructura de los isómeros del enalapril. Efecto de la temperatura de la columna en la forma de pico del enalapril.

Para alcanzar la sensibilidad necesaria para la determinación de los analitos a niveles de sub-ppb se requiere una etapa de tratamiento de la muestra. En este trabajo se escogió la extracción en fase sólida y se evaluaron los principales parámetros que afectan al proceso de extracción. En primer lugar, se estudió la eficacia de extracción de dos cartuchos. El sorbente del cartucho Oasis HLB es un copolímero compuesto por dos monómeros, divinilbenceno (lipofílico) y N-vinilpirrolidona (hidrofílico), que resulta adecuado tanto para los compuestos polares como apolares. En el caso de los cartuchos MCX, están diseñados especialmente para la retención de los compuestos básicos. En ambos casos el pH de la muestra debe ajustarse para que la retención de los analitos en el sorbente sea óptima.

Los compuestos en modo negativo mostraron un comportamiento similar en ambos cartuchos aunque, en general, las recuperaciones fueron más altas utilizando los HLB. En el caso de los compuestos analizados en modo positivo, el comportamiento fue dispar aunque para la mayoría de ellos la eficacia de extracción del cartucho HLB fue mayor. Por ello se seleccionó dicho cartucho (*Figura 1, artículo científico 1*).

Respecto a la elección del pH, las recuperaciones fueron más elevadas trabajando a pH 7, con la excepción de algunos compuestos como la atorvastatina, olanzapina y 4-aminoantipirina para los que no se obtuvieron resultados satisfactorios. En el caso de los dos primeros compuestos, el uso de sus isótopos deuterados utilizados desde el principio del proceso de extracción corrigió este problema.

Otro de los parámetros de los que depende la eficacia del proceso SPE es el disolvente de elución utilizado. En este trabajo se realizó una comparación entre tres disolventes orgánicos relativamente polares: metanol, acetonitrilo y acetona. Atendiendo a su poder de elución para desplazar solutos de un sorbente, ordenación conocida como serie eluotrópica, el metanol es el disolvente más polar (mayor poder eluyente), mientras que la acetona es el más apolar de los tres. Los resultados observados están de acuerdo con esta ordenación pues utilizando acetona los valores de recuperación fueron menores para dos de los compuestos. El disolvente que dio una mayor recuperación fue el metanol.

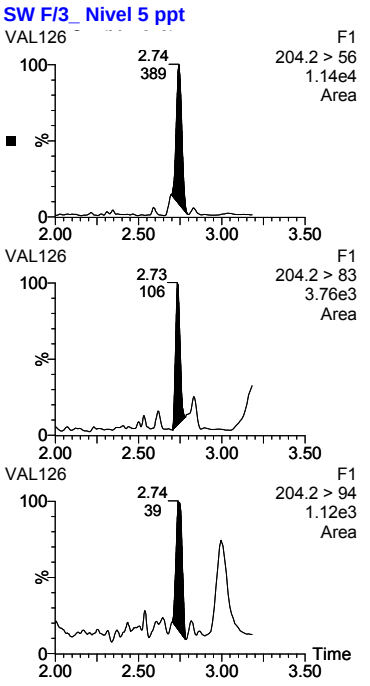
Los resultados obtenidos evidencian la complejidad que supone encontrar unas condiciones óptimas para todos los compuestos y la necesidad de alcanzar una situación de compromiso. Cuando se analiza un solo analito, o varios analitos pertenecientes a la misma familia química, se pueden elegir unas condiciones experimentales específicas que favorecen a todos ellos. En cambio, en una determinación multirresidual las condiciones de trabajo deben ser genéricas con el fin de extraer simultáneamente todos ellos, siendo necesario alcanzar una situación de compromiso. En ocasiones esto supone obtener resultados menos satisfactorios de lo esperado para algunos analitos.

Validación del método

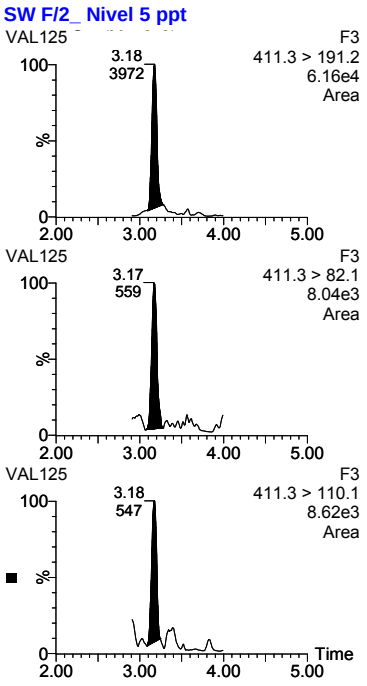
Se estudió la linealidad, exactitud, precisión, límite de detección (LOD) y límite de cuantificación (LOQ) en cada una de las matrices estudiadas. El estudio de la linealidad se realizó mediante la inyección por triplicado de once disoluciones patrón, en el rango comprendido entre 0.25 y 500 µg/L. Ocho de los compuestos mostraron una linealidad satisfactoria en todo el rango. Para otros seis compuestos su respuesta fue lineal entre 1 y 500 µg/L, y el resto de los compuestos, entre 5-500 µg/L. En todos los casos los residuales fueron inferiores al 30% y $r > 0.99$.

El método se validó mediante ensayos de recuperación en tres tipos de aguas (superficial, influente y efluente). Dichas muestras se fortificaron a distintos niveles en función de la sensibilidad de la combinación analito/matriz. En agua superficial se validó a 0.005, 0.025 y 0.05 µg/L; en efluente, a 0.1 y 0.5 µg/L y en influente, a 0.25 y 1.25 µg/L. En las *Tablas 2-4, artículo científico 1* se muestran los valores obtenidos correspondientes a la exactitud (% de recuperación) y a la precisión (desviación estándar relativa).

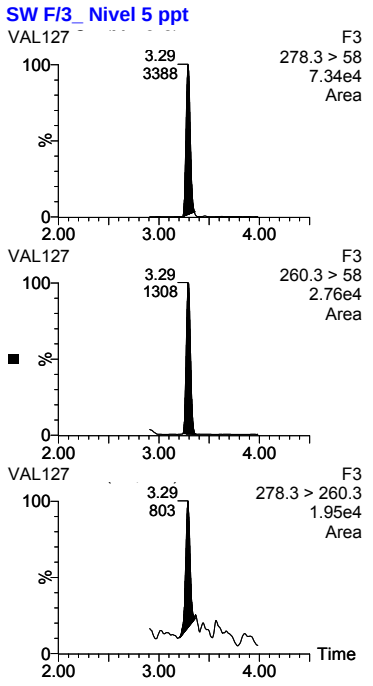
En agua superficial, ocho compuestos se validaron a 0.005 µg/L, detectando las tres transiciones seleccionadas para cada analito. Esto fue posible gracias a la gran sensibilidad para estos analitos. En la Figura 2.2 se muestran los cromatogramas de estos ocho compuestos.



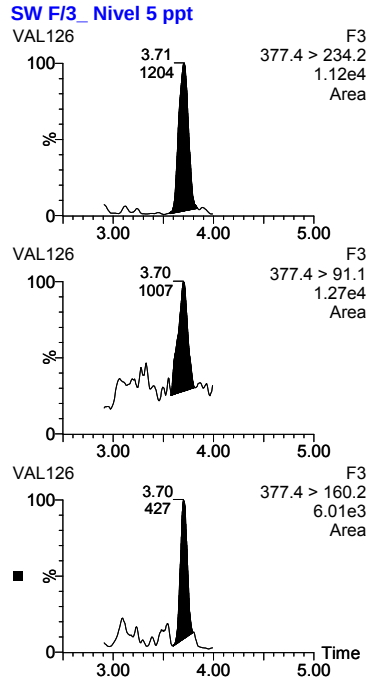
4-aminoantipyrine



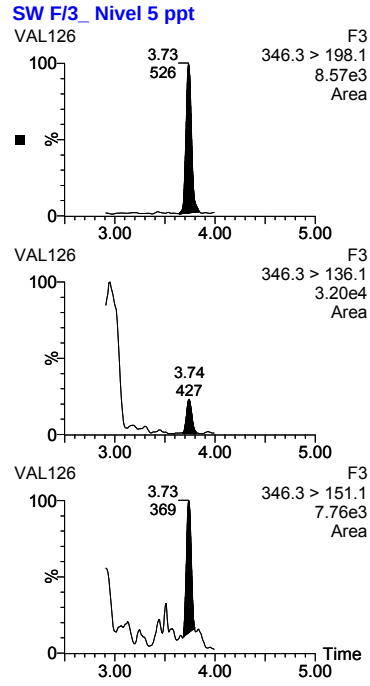
risperidone



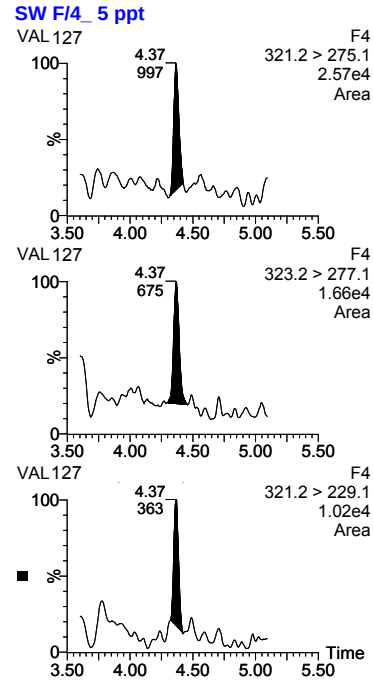
venlafaxine



enalapril



omeprazole



lorazepam

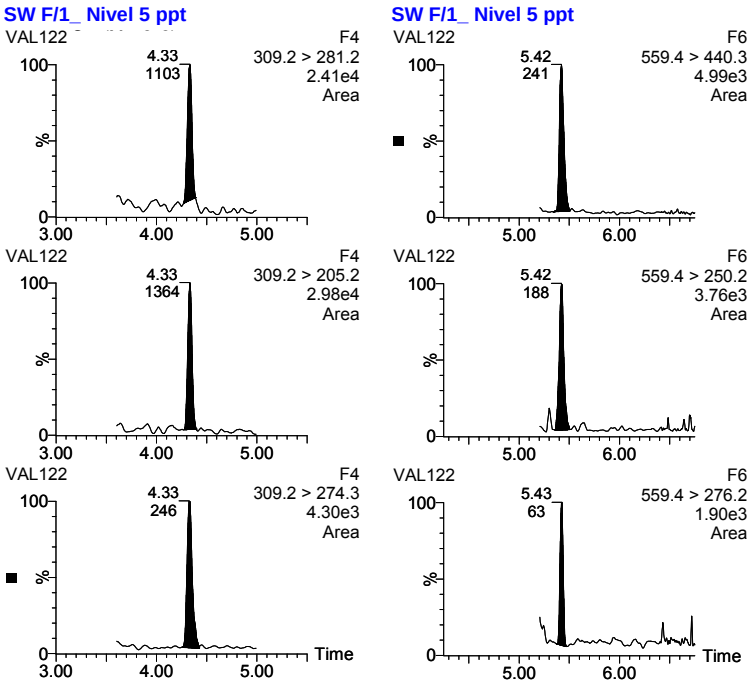


Figura 2.2 Cromatogramas UHPLC-MS/MS de los ocho compuestos validados a 0.005 µg/L.

En el caso del efluente, la validación al nivel más bajo (0.1 µg/L) fue satisfactoria para todos los compuestos excepto para cuatro de ellos. El motivo fue diverso: dos compuestos no se validaron debido a su baja respuesta al nivel estudiado, mientras que para los otros dos analitos la concentración encontrada en la muestra blanco utilizada impidió su cuantificación correcta a dicho nivel. Esta situación pone de manifiesto la dificultad de encontrar muestras "blanco" representativas para poder llevar a cabo la validación en el caso de efluentes urbanos. Este problema fue todavía mayor en la validación de influente. Ante la imposibilidad de obtener muestras blanco de dicha matriz, se realizó un análisis previo de varias muestras para estimar su concentración y se escogió aquella que contenía los niveles más bajos de los fármacos seleccionados. Además, la muestra se diluyó con el fin de reducir el contenido de materia orgánica y minimizar los interferentes presentes en la matriz. A pesar de ello, cuatro compuestos (4-aminoantipirina, naproxeno, atorvastatina e ibuprofeno) no se

pudieron validar al nivel más bajo debido a las elevadas concentraciones halladas en la muestra "blanco" seleccionada en la validación.

La presencia de ciertos compuestos seleccionados en las muestras empleadas en la validación también supuso una dificultad a la hora de calcular los valores de LOQ. En dichos casos este valor se determinó a partir del cromatograma de la muestra blanco, es decir, de la muestra no fortificada, teniendo en cuenta los niveles de concentración estimados en la misma.

Para contrarrestar el efecto matriz observado en las muestras procedentes de las EDAR, especialmente en el caso del influente, se utilizaron varios analitos marcados. Para aquellos compuestos para los que la corrección era necesaria y no disponíamos de su patrón marcado isotópicamente, la elección del patrón interno marcado se hizo en función del tiempo de retención. Como puede observarse en las *Tablas 3 y 4, artículo científico 1*, su uso condujo a una corrección satisfactoria en la mayoría de los analitos.

Análisis de muestras. Resultados en aguas superficiales, en influentes y en efluentes urbanos

La metodología desarrollada se aplicó a muestras de agua procedentes de tres EDAR distintas (42 muestras de influente y 42 de efluente).

Cabe destacar que la adquisición simultánea de tres transiciones por compuesto resultó muy útil a la hora de confirmar la identidad de algunos compuestos para los que se planteaba una cierta duda. Este fue el caso de una muestra que contenía bezafibrato en la que la relación de intensidad (*ion ratio*) de la primera transición de confirmación se encontraba fuera de los límites de tolerancia. En cambio, la segunda transición de confirmación cumplía el *ion ratio* (*Figura 2, artículo científico 1*). Por otro lado, en algunas muestras de influente el tiempo de retención de varios analitos se desviaba con respecto al del patrón. Sin embargo, la relación de intensidad entre la primera y la segunda transición estaba dentro del rango establecido. La adquisición de una segunda

transición de confirmación (q2) en la que también se cumplía el *ion ratio* nos permitió confirmar la presencia de estos compuestos (*Figuras 3 y 4, artículo científico 1*).

En relación a los resultados obtenidos, tan sólo tres compuestos no se detectaron en ninguna ocasión. Del resto, cabe destacar que el grupo de los analgésicos y antiinflamatorios, así como los fármacos utilizados para el tratamiento del colesterol, fueron los más frecuentemente detectados. Generalmente, las concentraciones en el influente fueron mayores que en el efluente, aunque hubo compuestos que únicamente se detectaron en las muestras de efluente. En el *artículo científico 3* se hará una discusión detallada de los resultados obtenidos.

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2.3 Determinación simultánea de fármacos, incluyendo numerosos antibióticos, en aguas medioambientales y residuales. Estudio y corrección del efecto matriz en el proceso de cuantificación

2.3.1 Introducción

Los antibióticos son el grupo de fármacos cuya presencia en el medio ambiente ha centrado mayor atención en los últimos años. Este hecho se debe principalmente a las elevadas cantidades que se consumen y al impacto que su presencia podría ocasionar en la salud humana y en el ecosistema acuático. En este sentido, la presencia de antibióticos en aguas medioambientales es preocupante ya que estos contaminantes contribuyen a la selección y propagación de cepas de resistencia bacteriana que podrían suponer un riesgo para las personas y los animales (Petrovic, 2005; Khetan, 2007; Hernández, 2007).

En todo el mundo se ha detectado un desarrollo creciente de resistencias bacterianas a casi todas las familias de antibióticos conocidas. Los mecanismos mediante los cuales las bacterias resisten a la acción de los antibióticos son complejos y su explicación excede los objetivos de esta Tesis. A grandes rasgos, se trata de un proceso de mutación y selección natural en el que las cepas que adquieren resistencia, es decir, aquellas que son capaces de adaptarse a los cambios en el medio ambiente, pueden sobrevivir y reproducirse, mientras que las cepas sensibles desaparecen. Dicho de otro modo, significa que las bacterias se vuelven inmunes a los antibióticos utilizados para tratar las infecciones.

La resistencia a algunos antibióticos puede producirse tras un tratamiento controlado, tras su uso prolongado y/o con posterioridad a la administración de antibióticos a concentraciones que resultan muy bajas para curar pero que son suficientemente altas para promover la aparición de cepas bacterianas resistentes (Moreno-Bondi, 2009).

El uso de los antibióticos en el ganado es una de las vías de proliferación de cepas de resistencia bacteriana y de bacterias patógenas. Las bacterias resistentes a los antibióticos pueden transferirse desde los animales hasta las personas a través del consumo de carne animal, provocando un impacto sobre la salud humana. Su uso es especialmente preocupante en aquellos casos en los que los antibióticos utilizados en la medicina veterinaria son los mismos que los que se emplean en los tratamientos de las

personas, o presentan estructuras muy similares. En este caso, las bacterias pueden desarrollar resistencia cruzada, es decir, el uso de un antibiótico puede aumentar los niveles de resistencia a ese compuesto y a otros similares (Batt, 2006; Seifrtová, 2009). Para minimizar esta ruta de transferencia de resistencia, las autoridades correspondientes de cada país supervisan el contenido de antibióticos en la carne (Kümmerer, 2009).

Las cepas resistentes también pueden transferirse a las personas mediante otras vías, por ejemplo, a través del agua, de los alimentos - si las plantas se riegan con agua superficial- o a través de los lodos procedentes de depuradoras que se utilizan como fertilizantes (Kümmerer, 2009). En cuanto a la presencia de bacterias resistentes a los antibióticos en el medio ambiente, se han encontrado en el agua superficial (Kümmerer, 2004; Watkinson, 2007), en el agua residual de las EDAR (Kümmerer, 2004; Schlüter, 2007) y en el suelo (Schmidt, 2008).

Sin embargo, con los datos disponibles actualmente no parece posible llegar a una conclusión sobre la importancia y el impacto que la resistencia bacteriana en el medio ambiente podría suponer en la salud y en el ecosistema. Del mismo modo, el impacto que los antibióticos presentes en las aguas puedan tener en el desarrollo de resistencia bacteriana plantea todavía muchas dudas (Kümmerer, 2009). Existe pues falta de información sobre el destino y los efectos de los antibióticos en el medio ambiente. Esta información resulta necesaria para poder evaluar sus posibles efectos negativos. Por ello, en esta Tesis se decidió estudiar este grupo de compuestos.

Otro de los motivos por los que el análisis de los antibióticos en el medio ambiente es importante es su elevado consumo. Se estima que anualmente se consumen a nivel mundial entre 100.000 y 200.000 toneladas (Wise, 2002). Es difícil realizar una comparación entre países sobre su consumo porque la información disponible en la literatura científica es escasa. Lo que sí parece claro es que al menos el 50% de los antibióticos se utilizan en la medicina humana. Así, según un estudio publicado en el año 2000 por la Federación Europea de Salud Animal (FEDESA), en 1999 se utilizaron en Europa y en Suiza más de 13.000 toneladas de antibióticos, de las

cuales el 65% se destinaron a la medicina humana, el 29% a la veterinaria y el resto como promotores del crecimiento. En el caso de Estados Unidos, de las cerca de 23.000 toneladas que se consumen anualmente, el 50% aproximadamente se utilizan para la cría de ganado, cerdos y aves de corral (Kümmerer 2009).

España figura entre los países de Europa que más antibióticos consume aunque es difícil establecer con precisión el consumo total ya que se estima que aproximadamente el 30% de los antibióticos más utilizados podrían obtenerse sin receta médica o bien de restos de tratamientos previos. A diferencia de otros países, en España se consumen sobre todo antibióticos de amplio espectro, que tienen mayor impacto en el desarrollo de resistencias (Vigilancia Europea del Consumo de Antimicrobianos, ESAC). Además, su consumo tiene un fuerte carácter estacional, concentrado en los meses de noviembre a febrero.

En este trabajo se han seleccionado alrededor de treinta antibióticos de amplio espectro que se han analizado junto con la lista de compuestos seleccionados en el *artículo científico 1*. El resultado ha sido el desarrollo de un método multirresidual para la cuantificación y confirmación simultánea de 47 compuestos. Todos los analitos se preconcentran simultáneamente mediante una etapa de extracción en fase sólida para alcanzar la sensibilidad necesaria. Finalmente, todos los compuestos se analizan en una única inyección mediante UHPLC-MS/MS. Como se ha comentado en el apartado 1.2.3, el desarrollo de un método de tales características conlleva una notable dificultad, sobre todo teniendo en cuenta el elevado número de compuestos analizados en el presente trabajo.

Se hizo especial hincapié en la correcta cuantificación de las muestras, especialmente en el caso de matrices complejas como la de efluente. Para ello se estudió la capacidad de corrección de una serie de patrones marcados isotópicamente (doce en concreto) en nueve aguas de efluente tomadas en diferentes depuradoras y en distintas épocas del año. Con ello pretendíamos comprobar la eficacia y la robustez del método desarrollado, es decir, evaluar la capacidad de corrección de los analitos marcados en

aguas de distinto origen y composición para poder extrapolar su uso al análisis de otras muestras distintas de efluente.

2.3.2 Artículo científico 2

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Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A multi-class method for the simultaneous quantification and confirmation of 47 pharmaceuticals in environmental and wastewater samples has been developed. The target list of analytes included analgesic and anti-inflammatories, cholesterol lowering statin drugs and lipid regulators, antidepressants, anti-ulcer agents, psychiatric drugs, ansiolitics, cardiovasculars and a high number (26) of antibiotics from different chemical groups. A common pre-concentration step based on solid-phase extraction with Oasis HLB cartridges was applied, followed by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) measurement. All compounds were satisfactorily determined in just one single injection, with a chromatographic run time of only 10 min. The process efficiency (combination of the matrix effect and the extraction process recovery) for the 47 selected compounds was evaluated in nine effluent wastewater (EWW) samples, and the use of different isotope-labelled internal standards (ILIS) was investigated to correct unsatisfactory values. Up to 12 ILIS were evaluated in EWW and surface water (SW). As expected, the ILIS provided satisfactory correction for their own analytes. However, the use of these ILIS for the rest of pharmaceuticals was problematic in some cases. Despite this fact, the correction with analogues ILIS was found useful for most of analytes in EWW, while was not strictly required in the SW tested. The method was successfully validated in SW and EWW at low concentration levels, as expected for pharmaceuticals in these matrices (0.025, 0.1 and 0.5 μg/L in SW; 0.1 and 0.5 μg/L in EWW). With only a few exceptions, the instrumental limits of detection varied between 0.1 and 8 pg. The limits of quantification were estimated from sample chromatograms at the lowest spiked levels tested and normally were below 20 ng/L for SW and below 50 ng/L for EWW. The developed method was applied to the analysis of around forty water samples (river waters and effluent wastewaters) from the Spanish Mediterranean region. Almost all the pharmaceuticals selected in this work were detected, mainly in effluent wastewater. In both matrices, analgesics and anti-inflammatories, lipid regulators and quinolone antibiotics were the most detected groups.

1. Introduction

In recent years, there has been a growing interest to investigate the impact on the environment of a wide group of compounds so-called "emerging" or "new" unregulated contaminants. Under this expression, different groups of analytes that are considered of concern for the environment are included (algal and cyanobacterial toxins, nanomaterials, drugs of abuse, surfactants, disinfection by-products, hormones and other endocrine disrupting compounds, pharmaceuticals and personal care products, etc.) and their presence has been investigated in different environmental matrices [1]. Their consumption around the world is continuously increasing and they are normally detected in environmental and urban wastewater.

In contrast to other compounds, e.g. pesticides, emerging contaminants are still not regulated in the environment to guarantee the quality of the water. Among the wide group of emerging contaminants, pharmaceuticals are one of the major concern (especially antibiotics) because of their wide consumption and their potential negative effect on the water quality and living organisms. The improvement of analytical methodologies in terms of sensitivity, selectivity and scope of the method is of great interest to have realistic reliable data on their presence in the environment.

After human and/or veterinary consumption, pharmaceuticals are excreted mainly in unchanged form as the parent compound, although many of them are partially metabolized. Consequently,

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ABSTRACT

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Keywords

Pharmaceuticals; Ultra-high performance liquid chromatography; Tandem mass spectrometry; Multi-class analysis; Surface water and wastewater; Matrix effects.

1. Introduction

In recent years, there has been a growing interest to investigate the impact on the environment of a wide group of compounds so-called “emerging” or “new” unregulated contaminants. Under this expression, different groups of analytes that are considered of concern for the environment are included (algal and cyanobacterial toxins, nanomaterials, drugs of abuse, surfactants, disinfection by-products, hormones and other endocrine disrupting compounds, pharmaceuticals and personal care products, etc.) and their presence has been investigated in different environmental matrices [1]. Their consumption around the world is continuously increasing and they are normally detected in environmental and urban wastewater.

In contrast to other compounds, e.g. pesticides, emerging contaminants are still not regulated in the environment to guarantee the quality of the water. Among the wide group of emerging contaminants, pharmaceuticals are one of the major concern (especially antibiotics) because of their wide consumption and their potential negative effect on the water quality and living organisms. The improvement of analytical methodologies in terms of sensitivity, selectivity and scope of the method is of great interest to have realistic reliable data on their presence in the environment.

After human and/or veterinary consumption, pharmaceuticals are excreted mainly in unchanged form as the parent compound, although many of them are partially metabolized. Consequently, both parents and metabolites enter into urban wastewater and are matter of

concern from the analytical point of view. Most of these compounds are not completely removed during wastewater treatments and they can finally arrive at surface and ground waters [2]. The low pharmaceutical concentrations typically present (low ng/L) seem not to cause adverse effects on humans and in the aquatic environment, but not reliable data are currently available about long-term risk derived from their continuous input in the natural environment. Among pharmaceuticals, the presence of antibiotics in water causes more concern because they can induce bacterial resistance, even at low concentrations, through their continuous exposure [3] and [4]. Recent studies have reported that quinolone and fluoroquinolone cause the development of genotoxicity based on an*in vitro* bioassay [5] and the quinolone ciprofloxacin has effects on plankton and algae grown at environmental relevant concentrations [1].

Highly sensitive methods are required to determine the low levels normally present in environmental matrices. At present, the combination of ultra high-performance (or pressure) liquid chromatography (UHPLC) with (fast) tandem MS is surely the most suitable approach nowadays. This hyphenated technique provides the sensitivity and selectivity required in this type of analysis. The growing trend on using UHPLC coupled with MS/MS can be inferred from the evolution of scientific papers published during the last decade [6]. Recently, the use of UHPLC–QTOF MS has been proposed for rapid screening of antibiotics. This technique has been proven to be an efficient approach for detection and safe identification of these compounds in water [7].

Despite the high sensitivity reached by MS/MS analyzers, the majority of applications still need a pre-concentration step for the accurate analyte determination at sub-ppb levels. In the vast majority of methods, off-line solid phase extraction (SPE) mode is applied for this purpose [2, 8-12], although automated on-line SPE coupled to LC–MS is an increasing trend [8, 13-15].

Most of methods recently developed pursue the simultaneous determination of multi-class compounds because many compounds from very different therapeutical classes are found when monitoring environmental waters [4,9,16]. Obviously, multi-class methods provide more information about the occurrence of pharmaceuticals than single group analysis, with reduced analysis time and cost. However, the development of these methods involves a compromise in the selection of experimental conditions (i.e. LC separation, MS detection and sample preparation) [8]. Firstly, the LC chromatographic conditions should be optimized to enhance resolution and to minimize undesired co-elution. Secondly, a compromise between sensitivity and selected dwell times should be found to maintain satisfactory peak shape for all selected

compounds. For this purpose, the MS/MS method is usually divided into different elution-time windows that contain different selected reaction monitoring (SRM) transitions with appropriate dwell times [6]. However, this restriction is nowadays changing as recent triple quadrupole instruments allow working with dwell times as low as of 0.001 s without affecting the method sensitivity. Moreover, new acquisition softwares make this task easier selecting automatically the most suitable dwell time for each compound based on time overlapping and chromatographic peakwidth. Finally, the sample procedure applied should assure the simultaneous efficient recovery of all selected compounds. This aspect is problematic in wide-scope multi-class methods, as pharmaceuticals belonging to different therapeutical groups can have rather different physico-chemical properties. As extraction efficiency is affected by several variables such as the type of sorbent used, sample pH or sample volume loaded, it has to be carefully tested for successful results. Finally, a satisfactory compromise should be reached along the overall analytical method for the simultaneous analysis of all target compounds.

A drawback associated to LC–MS/MS methods deals with matrix effects, which are attributed to the presence of undesirable sample components that co-elute with the analytes altering the ionization process. The consequence of matrix effects is the suppression or enhancement of the signal, which can affect both identification and quantification of analytes. Matrix effects depend on each analyte/matrix combination, but also on the sample preparation applied, the chromatographic separation, mass spectrometry instrumentation and the ionization conditions [17]. It is not possible to predict whether the combination of these conditions will affect the analyte signal or not; therefore, the evaluation of matrix effect should be included in the validation process of the method considering the different matrices studied (e.g. surface water, effluent water and influent water). Several strategies have been proposed to solve matrix effects, including modifications of the sample pre-treatment, the chromatographic or MS conditions and the calibration techniques [17]. In the field of antibiotics analysis, some of these approaches have being reviewed by our own group [18]. The use of isotope-labelled internal standards (ILIS) is, by far, the most used to face pharmaceutical analysis [10-12].

The goal of this paper is to develop a rapid, accurate and sensitive analytical strategy based on the use of UHPLC–MS/MS for the simultaneous determination (quantification and confirmation) of 47 pharmaceuticals that belong to the most representative therapeutical groups. A high number of antibiotics (around 30) have been added to the target list of our previous method [10] in order to have wider and more realistic knowledge of the presence of

pharmaceuticals in the environment. Several ILIS have been tested to correct unsatisfactory values associated to matrix effects and/or potential losses associated to the SPE step.

2. Experimental

2.1. Reagents and chemicals

Reference standards of acetaminophen (paracetamol), salicylic acid, ibuprofen, 4-aminoantipyrine, omeprazole, ketoprofen, naproxen, bezafibrate, diclofenac, gemfibrozil, pravastatin sodium and enalapril maleate salt were purchased from Sigma–Aldrich (St Louis, MO, USA). Pantoprazole, lorazepam, alprazolam, venlafaxine hydrochloride, risperidone, simvastatin and paroxetine hydrochloride were from LGC Promochem (London, UK). Atorvastatin and olanzapine were supplied by Toronto Research Chemicals (Ontario, Canada). Antibiotic reference standards of sulfamethoxazole, sulfamethazine, sulfadiazine and sulfathiazole were from Across Organics (Geel, Belgium). Enrofloxacin, moxifloxacin and ciprofloxacin were from Bayer Hispania (Barcelona, Spain). Sarafloxacin, marbofloxacin and pefloxacin were provided by Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid Spain) and Aventis Pharma (Madrid, Spain), respectively. The rest of antibiotics were supplied by Sigma–Aldrich. All reference standards presented purity higher than 93%.

Isotopically labelled compounds of omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, salicylic acid-d_3 and ibuprofen-d_3 were from CDN Isotopes (Quebec, Canada); atorvastatin-d_5, paroxetine hydrochloride-d_4 and olanzapine-d_3 from Toronto Research Chemicals; sarafloxacin-d_8 hydrochloride trihydrate from Sigma–Aldrich and sulfamethoxazole-$^{13}C_6$ and trimethoprim-$^{13}C_3$ were from Cambridge Isotope Laboratories (Andover, MA, USA).

HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained from distilled water passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac, reagent grade) and sodium hydroxide (NaOH, >99%) were supplied by Scharlab (Barcelona, Spain).

Individual stock solutions of pharmaceuticals were prepared dissolving 25 mg, accurately weighted, in 50 mL methanol, obtaining a final concentration of 500 mg/L. For antibiotics, individual stock solutions were prepared dissolving 50 mg of solid standard in 100 mL ACN, except quinolones that were dissolved in MeOH. The addition of 100 µL of 1 M NaOH

was necessary for the proper dissolution of the acidic analytes like quinolones. Stock solutions were stored at −20 °C.

Individual stock solutions of ILIS were prepared in methanol. A mix working solution at 100 µg/L (for those ionizing in positive mode) and at 1 mg/L (for ILIS ionizing in negative mode) was prepared in MeOH and used as surrogate.

An intermediate mixed solution containing all antibiotics at a concentration of 5 mg/L was obtained after mixing individual stock solutions and diluting with MeOH. Another intermediate solution containing the rest of pharmaceuticals was prepared also in MeOH following a similar procedure. From intermediate solutions, a mixture of all compounds at a concentration of 500 µg/L was prepared in MeOH. Working solutions were subsequently prepared from the mixed solution by diluting the appropriate volume with HPLC-grade water. All standard solutions (stock, intermediate and working solutions) were stored in amber glass bottles at −20 °C in a freezer. Cartridges used for SPE were Oasis HLB (60 mg) from Waters (Milford, MA, USA).

2.2. Liquid chromatography

UHPLC analysis were carried out using an Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was performed using an Acquity UPLC HSS T3 column, 1.8 µm, 100 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 60 °C and the sample manager was maintained at 5 °C. Mobile phase consisted of a water/methanol, both 0.1 mM NH_4Ac and 0.01% HCOOH, gradient. The methanol percentage was changed linearly as follows: 0 min, 5%; 7 min, 90%; 8 min, 90%; 8.1 min; 5%. Analysis run time was 10 min. The sample injection volume was 20 µL.

2.3. Mass spectrometry

A TQD (triple quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters Corp., Milford, MA, USA) was used. Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N_2 LC–MS (Claind, Teknokroma, Barcelona, Spain). The cone gas and the desolvation gas flows were set at 60 L/h and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair,

Valencia, Spain) with a performance of 2×10^{-3} mbar in the T-Wave collision cell. Capillary voltages of −3.0 kV (negative ionization mode) and 3.5 kV (positive ionization mode) were applied. The interface temperature was set to 500 °C and the source temperature to 120 °C. A scan time of 0.01 s was selected.

Masslynx 4.1 (Micromass, Manchester, UK) software was used to process quantitative data.

2.4. Recommended procedure

The SPE method was based on our previous work developed for the determination of 20 pharmaceuticals [10]. The procedure was as follows: 100 mL water sample were spiked with the ILIS mix working solution to give a final concentration of 0.1 µg/L for each ILIS determined in positive mode and of 1 µg/L for those ILIS determined in negative mode. Oasis HLB (60 mg) cartridges were conditioned with 3 mL MeOH and 3 mL HPLC-grade water before use. Then, samples were passed through the cartridge and, after drying under vacuum, analytes were eluted with 5 mL methanol. The extract was evaporated to dryness under a gentle nitrogen stream at 40 °C and reconstituted with 1 mL MeOH–water (20:80, v/v). Finally, 20 µL were injected in the UHPLC–MS/MS system under the conditions shown in Table 1. Quantification was made using calibration standards prepared in solvent, based on relative responses analyte/ILIS, or on absolute responses, depending on whether ILIS was used for correction or not. ILIS were used to correct for matrix effects and/or SPE potential errors as shown in Tables 2-4.

2.5. Validation study

Method accuracy (estimated by means of recovery experiments) and precision (expressed as repeatability in terms of relative standard deviation (RSD)) were studied by recovery studies in surface water (SW) and effluent wastewater (EWW) spiked at different concentrations (25, 100 and 500 ng/L for SW; 100 and 500 ng/L for EWW). All experiments were performed in quintuplicate. Recovery values between 70% and 120%, with RSD lower than 20% were considered as satisfactory.

The limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio of 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. Rearding EWW, adequate blanks samples were not found for several analytes. In these cases, LOQ values were estimated from quantified levels present in non-spiked blanks. The instrumental limit of detection (LOD) was estimated for S/N = 3 from the chromatograms of standards at the lowest concentration level tested in the calibration curve.

The linearity of the method was studied by analyzing standard solutions in triplicate at seven concentrations in the range from 1 to 100 µg/L. Satisfactory linearity using weighed (1/*X*) least squares regression was assumed when the correlation coefficient (*r*) was higher than 0.99 and residuals lower than 30% without significant trend, based on relative responses (analyte peak area/ILIS peak area), except for those compounds that were quantified without ILIS (absolute response).

2.6. Application to real samples

Around 40 samples of SW (18 samples) and EWW (19 samples) were collected in polyethylene high-density bottles in selected sites of the Spanish Mediterranean area (Castellon and Valencia provinces). Samples were stored at <−18 °C until analysis. Before analysis, samples were thawed at room temperature. Wastewater samples consisted on 24-h composite urban wastewater samples and were collected from different wastewater treatment plants (WWTPs).

3. Results and discussion

In this work, 47 pharmaceuticals from the most representative therapeutical groups were studied (see Table 1). Among them, 21 compounds are widely consumed in human medicine in Spain [19]. The rest are antibiotics and were selected due to their potential negative effect on living organisms of the aquatic environment. All analytes corresponded to parent compounds except salicylic acid and 4-aminoantipyrine, the metabolites of acetylsalicylic acid and dipyrone, respectively. These metabolites were selected based on information reported in scientific literature on their occurrence in surface and wastewater [11, 20-22] and on the human metabolism of their parent pharmaceuticals [23-25].

Table 1
MS/MS optimized conditions for selected compounds.

Compound	Therapeutic group	Polarity (ES)	LOD (pg)	MW	Q Transition	Cone (V)	C.E.(eV)	q_1 Transition	C.E.(eV)	Q/q
Acetaminophen	Analgesic and anti-inflammatories	+	3.9	151.1	152.1 > 110.1	30	15	152.1 > 93.0	25	7.5
4-Aminoantipyrine		+	0.2	203.3	204.2 > 56.0	30	20	204.2 > 83.0	15	9.6
Diclofenac		-	5.9	295.0	294.1 > 250.1	30	10	296.1 > 252.1	30	1.3
Ibuprofen		-	86.0	206.1	205.1 > 161.1	30	10	-	-	-
Ketoprofen		-	7.4	254.1	253.2 > 209.2	20	5	-	-	-
Naproxen		-	6.6	230.1	185.2 > 170.1	30	10	229.2 > 170.1[a]	20	7.8
Salicylic acid		-	56.6	138.0	137.1 > 93.0	30	15	-	25	-
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	+	1.2	558.3	559.4 > 440.3	45	20	559.4 > 250.2	45	0.9
Simvastatin		+	1.0	418.3	419.5 > 285.3	30	10	419.5 > 199.2	20	1.7
Pravastatin		-	18.5	424.2	423.4 > 321.2	40	15	423.4 > 101.1	30	1.3
Bezafibrate		-	1.8	361.1	360.2 > 274.1	30	15	362.2 > 276.2	20	2.2
Gemfibrozil		-	3.6	250.2	249.3 > 121.0	30	15	249.3 > 127.0	10	14.6
Paroxetine	Antidepressants	+	3.8	329.1	330.3 > 70.1	50	20	330.3 > 44.1	30	0.6
Venlafaxine		+	0.4	277.2	278.3 > 58.0	30	15	278.3 > 260.3	15	1.2
Omeprazole	Anti-ulcer agents	+	0.7	345.1	346.3 > 198.1	30	10	346.3 > 136.1	35	2.4
Pantoprazole		+	0.3	383.1	384.3 > 138.1	25	10	384.3 > 200.2	35	1.3
Olanzapine	Psychiatric drugs	+	1.6	312.1	313.3 > 256.2	45	25	313.3 > 84.1	25	1.3
Risperidone		+	0.6	410.2	411.3 > 191.2	50	30	411.3 > 82.1	60	16.8
Alprazolam	Ansiolitics	+	0.7	308.1	309.2 > 281.2	60	25	309.2 > 205.2	40	1.2
Lorazepam		+	2.7	320.2	321.2 > 275.1	40	20	323.2 > 277.1	20	1.2
Enalapril	Cardiovasculars	+	0.3	376.2	377.4 > 234.2	35	20	377.4 > 91.1	55	1.2
Erithromycin	Macrolide antibiotics	+	0.1	733.5	734.4 > 158.1	35	30	734.4 > 576.3	25	3.9
Clarithromycin		+	0.7	747.5	590.3 > 158.1	55	25	748.3 > 158.1[b]	30	1.5
Tylosin		+	0.2	915.5	916.9 > 174.2	50	35	916.9 > 101.0	40	4.2
Roxithromycin		+	0.2	836.5	679.8 > 158.1	60	30	837.9 > 158.1[b]	40	1.1
Moxifloxacin	Quinolone antibiotics	+	5.2	401.2	402.3 > 364.3	35	25	402.3 > 384.3	20	0.2
Norfloxacin		+	2.2	319.1	320.1 > 276.1	45	20	320.1 > 302.1	20	0.4
Pefloxacin		+	13.0	333.1	334.4 > 233.4	45	25	334.4 > 316.4	20	0.5
Ofloxacin		+	0.9	361.1	362.1 > 318.1	45	20	362.1 > 261.0	30	0.9
Marbofloxacin		+	13.5	362.1	363.4 > 320.4	35	20	363.4 > 345.4	20	0.7
Ciprofloxacin		+	28.9	331.1	332.1 > 231.1	45	40	332.1 > 314.1	20	1.3
Enrofloxacin		+	1.3	359.2	360.4 > 245.4	45	25	360.4 > 316.4	20	0.6
Sarafloxacin		+	4.4	385.1	386.4 > 299.3	40	30	386.4 > 368.4	20	0.7
Flumequine		+	0.6	261.1	262.3 > 202.3	35	30	262.3 > 244.3	20	0.5
Oxolinic acid		+	0.7	261.2	262.3 > 244.3	35	20	262.3 > 216.2	30	162
Nalidixic acid		+	0.4	232.1	233.2 > 187.2	20	25	233.2 > 215.2	20	0.8
Pipedimic acid		+	13.6	303.1	304.0 > 217.0	45	25	304.0 > 276.0	20	0.5
Sulfamethoxazole	Sulfonamide antibiotics	+	0.2	253.1	254.0 > 91.9	40	30	254.0 > 155.9	20	2.1
Sulfamethazine		+	0.2	278.1	279.3 > 92.0	40	30	279.3 > 186.2	15	0.7
Sulfadiazine		+	0.3	250.1	251.2 > 65.1	30	50	251.2 > 92.0	20	1.9
Sulfathiazole		+	0.5	255.0	256.2 > 156.0	30	15	256.2 > 92.0	25	1.3
Lincomycin	Lincosamide antibiotics	+	0.4	406.2	407.1 > 126.1	40	30	407.1 > 359.2	20	17
Clindamycin		+	0.6	424.2	425.1 > 126.0	45	30	427.1 > 126.0	25	4.5
Furaltadone	Other antibiotics	+	0.2	324.1	325.3 > 100.2	25	30	325.3 > 281.3	10	2.1
Furazolidone		+	0.6	225.0	226.3 > 139.2	35	15	226.3 > 122.1	20	1.2
Trimethoprim		+	0.8	290.1	291.1 > 230.1	50	25	291.1 > 261.1	25	1.8
Chloramphenicol		-	5.5	322.0	321.3 > 152.3	30	15	321.3 > 257.1	10	1.6
Acetaminophen-d_4		+	-	155.1	156.1 > 114.1	35	20	-	-	-
Diclofenac–d_4		-	-	299.0	300.1 > 256.1	30	10	-	-	-
Ibuprofen-d_3		-	-	209.1	208.2 > 164.2	20	10	-	-	-
Salicylic acid-d_4		-	-	142.1	141.1 > 97.0	30	20	-	-	-
Atorvastatin-d_5		+	-	563.3	564.4 > 445.2	45	25	-	-	-
Simvastatin-d_6		+	-	424.3	425.5 > 285.3	20	10	-	-	-
Paroxetine-d_4		+	-	333.2	334.3 > 74.1	40	30	-	-	-
Olanzapine-d_3		+	-	315.2	316.3 > 256.2	45	25	-	-	-
Omeprazole-d_3		+	-	348.1	349.1 > 198.1	30	10	-	-	-
Sarafloxacin-d_8		+	-	393.2	394.4 > 303.3	35	30	-	-	-
Sulfamethoxazole-$^{13}C_6$		+	-	259.2	260.2 > 98.2	30	30	-	-	-
Trimethoprim-$^{13}C_3$		+	-	293.3	294.1 > 233.1	40	20	-	-	-

ES, electrospray ionization; MW, monoisotopic molecular weight; Q, quantification; q, confirmation; C.E., collision energy.
[a] In this case an in-source fragment was used as precursor ion and the cone voltage was lowered to 20 V.
[b] In this case an in-source fragment was used as precursor ion and the cone voltage was lowered to 40 V.

3.1. MS and MS/MS optimization

Full-scan and MS/MS mass spectra of analytes were obtained from infusion of 1 mg/L methanol/water (50:50, v/v) individual standard solutions at a flow rate of 10 µL/min. The compounds investigated belong to various chemical groups and showed rather different ionization behaviour. The majority of the compounds (38 out of 47) were determined under positive ionization and the rest (9 out of 47) under negative ionization. All compounds showed an abundant $[M+H]^+$ or $[M-H]^-$ ion, except naproxen, roxithromycin and clarithromycin that showed better sensitivity when using an in-source fragment as precursor ion by increasing the cone voltage.

The two most sensitive SRM transitions were selected for each compound: the most abundant was used for quantification (Q) whereas the second one was for confirmation (q). This allowed us to reach the minimum number of identification points (IPs) required (3 IPs for legally registered compounds) for a safe confirmation [26]. Only one transition could be monitored for ibuprofen, salicylic acid and ketoprofen, due to their poor fragmentation.

Non-specific transitions (i.e. loss of water) were avoided in order to improve the selectivity of the method and to decrease the possibilities for occurrences of false positives or false negatives. Thus, for quinolone antibiotics, although the transition corresponding to the neutral loss of H_2O was the most sensitive, the second most sensitive transition was selected for quantification (Q), i.e. $[M+H-CO_2-C_2H_5N]^+$ for sarafloxacin and pipedimic acid, $[M+H-H_2O-C_2H_5N-C_3H_4]^+$ for pefloxacin and ciprofloxacin, and $[M+H-CO_2]^+$ for the rest of quinolones, in similarity to previous works [5,15]. The exception was oxolinic acid, where the $[M+H-H_2O]^+$ ion was chosen for quantification because it was much more sensitive than the rest.

For sulfonamide antibiotics, the *m*/*z* = 92 product ion, corresponding to the amide ring ($NH_2-C_6H_4$), was chosen for the four compounds belonging to this therapeutic group. This ion was the most abundant fragment in the case of sulfamethazine and sulfamethoxazole, while for sulfadiazine and sulfathiazole it was selected for confirmation. This product ion has been already reported by other authors [27,28].

Most of analgesic and anti-inflammatory compounds were normally in ESI negative mode. For these compounds, the neutral loss of CO_2 $[M-H-44]^-$ was the main product ion observed.

Regarding ILIS, only one transition was monitored. In the particular case of diclofenac-d_4, the transition 300.1 > 256.1 was chosen in order to avoid the mass overlap between the natural analyte (isotope peak due to the presence of two chlorine atoms; $2Cl^{37}$) and the ILIS signal, which would have occur if the transition 298.1 > 254.1 was chosen.

Dwell times of 10 ms were selected to assure enough data points per chromatographic peak (at least 10 points) to have satisfactory peak shape. All SRM transitions (around 100) were divided along eight overlapping windows. This favourable overlapping was possible due to the low positive-to-negative-switching time (20 ms) attainable by the triple quadrupole analyzer used in this work.

Mass spectrometry parameters selected, precursor and product ions, and instrumental LODs are shown inTable 1.

3.2. Chromatographic conditions

In this work, a UPLC HSS column (100 mm × 2.1 mm, 1.8 μm) was chosen for the separation of 59 compounds (47 analytes and 12 internal standards) in only 10 min. A larger column, compared to our previous work [10], was required for a satisfactory separation of higher number of analytes but maintaining similar chromatographic runs. As target compounds belong to different groups and have quite distinct physico-chemical characteristics, with different ionization behaviour (e.g. sensitivity for analytes determined in positive mode was normally better than in negative mode), it was necessary to find a compromise for their satisfactory separation using the same mobile phase. Methanol and acetonitrile with different modifiers (HCOOH and NH_4Ac at various concentrations) were tested for this purpose. A mobile phase containing both 0.1 mM NH_4Ac and 0.01% HCOOH, which was also used for the chromatographic separation of 20 pharmaceuticals [10] led to good peak shape and sensitivity for the wide majority of compounds. Therefore, this mobile phase (see Section 2.2) was selected as a compromise for the simultaneous chromatographic separation of both positive and negative ionized analytes.

3.3. Method validation

The linearity of the method was studied in the range 1–100 μg/L for all selected compounds. These values corresponded to 0.01–1 μg/L in the water samples taking into

account the pre-concentration factor applied along the sample procedure. Calibration curves showed satisfactory correlation coefficients (greater than 0.99) and residuals were lower 30% for all compounds.

Instrumental LODs are shown in Table 1. For the majority of the compounds (24 out of 47) LODs were below 1 pg, and for 20 analytes varied from 1 to 20 pg. In the case of ciprofloxacin, the LOD could have been improved if the non-specific transition corresponding to the loss of water (the most sensitive) had been selected instead of 332 > 231 that was finally used for quantification. The LODs for ibuprofen and salicylic acid were higher notably than for the rest of compounds. The reason was their poor fragmentation, and that only a low sensitive transition could be monitored.

It is well known that matrix effects are one of the main drawbacks of LC–MS/MS methods when applied to environmental samples. These effects may considerably alter the signal of many analytes, affecting severely to the quantification process. These effects are more noticeable when analysing complex-matrix samples like wastewater. A detailed study of those variables that may affect the overall analytical process efficiency (i.e. the matrix effect and the extraction process) is required when an analytical method is developed. In the line of our previous works on LC–MS/MS analysis of wastewater samples [29] we have evaluated the overall process efficiency for the 47 selected compounds. The process efficiency (PE) represents the percentage of matrix effect (ME) and extraction process recovery (RE), and it is expressed as [30]:

$$PE(\%) = \frac{ME(\%) \cdot RE(\%)}{100}$$

For this purpose, nine different EWW samples collected from three different WWTPs of the Castellon province (sample collection performed in autumn, winter and spring) were spiked at 500 ng/L for each individual compound as well as with the ILIS mix working solution (12 ILIS). “Blank” EWW samples, spiked only with the ILIS mix, were also processed to subtract the responses of possible target compounds. Their relative responses were quantified by internal standard calibration with standards in solvent.

Table 2 shows the average overall process efficiency for the nine samples, as well as the average RSD value before and after correction with an ILIS. For the wide majority of

Table 2
Average process efficiency and RSD values obtained from nine different EWW samples, spiked at 500 ng/L level and collected from three WWTPs.

Compound	Polarity (ES)	t_R (min)	Before correction		After correction		
			Process efficiency (%)	RSD (%)	Process efficiency (%)	RSD (%)	ILIS used
Acetaminophen	+	2.40	26	18	103	3	Acetaminophen-d_4
Sulfadiazine	+	2.51	14	26	50	25	Acetaminophen-d_4
Furaltadone	+	2.55	59	23	68	16	Trimethoprim-$^{13}C_3$
Sulfathiazole	+	2.59	68	18			-
Pipedimic acid	+	2.84	36	23	104	22	Acetaminophen-d_4
Marbofloxacin	+	2.97	70	25	117	9	Sulfamethoxazole-$^{13}C_6$
Trimethoprim	+	2.98	80	13	85	11	Trimethoprim-$^{13}C_3$
Lincomycin	+	3.00	88	13			-
Olanzapine	+	3.14	82	28	97	15	Olanzapine-d_3
Ofloxacin	+	3.15	51	26	89	25	Sulfamethoxazole-$^{13}C_6$
Pefloxacin	+	3.19	101	20			-
Norfloxacin	+	3.20	40	17			-
Furazolidone	+	3.23	43	26	84	18	Sulfamethoxazole-$^{13}C_6$
Ciprofloxacin	+	3.30	37	25	98	19	Sulfamethoxazole-$^{13}C_6$
Enrofloxacin	+	3.43	128	14	108	12	Sarafloxacin-d_8
4-Aminoantipyrine[a]	+	3.43	-	-			-
Sulfamethazine	+	3.46	96	13			-
Sulfamethoxazole	+	3.60	41	25	84	7	Sulfamethoxazole-$^{13}C_6$
Sarafloxacin	+	3.65	60	18	72	13	Sarafloxacin-d_8
Salicylic acid	-	4.32	22	6	96	15	Salicylic acid-d_4
Moxifloxacin	+	4.40	108	33			-
Chloramphenicol	-	4.48	70	14			-
Risperidone	+	4.50	144	11	122	5	Sarafloxacin-d_8
Venlafaxine	+	4.62	165	7			-
Clindamycin	+	5.14	90	10			-
Enalapril	+	5.35	83	10			-
Paroxetine	+	5.39	89	24	99	8	Paroxetine-d_4
Nalidixic acid	+	5.45	64	20			-
Oxolinic acid	+	5.55	47	21	106	12	Atorvastatin-d_5
Flumequine	+	5.55	35	25	83	14	Atorvastatin-d_5
Omeprazole	+	5.56	129	14	115	3	Omeprazole-d_3
Tylosin	+	5.72	55	11	112	21	Sulfamethoxazole-$^{13}C_6$
Erithromycin	+	5.74	54	17	114	22	Sulfamethoxazole-$^{13}C_6$
Pantoprazole	+	5.80	99	14			-
Pravastatin	-	6.12	55	17	91	18	Diclofenac–d_4
Clarithromycin	+	6.26	43	12	82	18	Sulfamethoxazole-$^{13}C_6$
Roxithromycin	+	6.32	70	14			-
Ketoprofen	-	6.32	37	19	55	16	Diclofenac–d_4
Lorazepam	+	6.40	89	11			-
Alprazolam	+	6.46	60	14			-
Naproxen	-	6.47	35	21	55	17	Diclofenac–d_4
Bezafibrate	-	6.56	56	15	94	17	Diclofenac–d_4
Atorvastatin	+	6.99	45	13	106	5	Atorvastatin-d_5
Diclofenac	-	7.19	49	25	86	16	Diclofenac–d_4
Ibuprofen	-	7.35	86	12	116	6	Ibuprofen-d_3
Gemfibrozil	-	7.75	42	18	71	2	Diclofenac–d_4
Simvastatin	+	8.13	37	25	100	8	Simvastatin-d_6

ES, electrospray ionization; t_R retention time.

[a] Not estimated due to the high analyte levels found in the "blank" samples.

compounds, PE < 100% were obtained. This may be due to matrix effects (ion suppression) and/or compound losses during SPE process. A few compounds showed PE > 100%, which was surely due to matrix effects resulting in ionization enhancement, as it is not expected to obtain RE > 100% due to the presence of compounds released from the SPE cartridges and coeluting with the analytes producing signal enhancement. In the case of 4-aminoantipyrine, data could not be reported due to high concentrations found in the samples tested.

As only 14 out of 47 pharmaceuticals showed satisfactory recoveries (without using any ILIS), it seems clear that some correction is required to obtain successful results. Otherwise, non-accurate quantification would be made leading typically to concentrations lower than actually present in the samples. The use of ILIS is nowadays widely accepted for matrix effects correction in environmental and wastewater analysis. However, the large number of compounds analyzed in our multi-residue method made unfeasible to correct each analyte with its own ILIS. Then, we considered the possibility of correcting unsatisfactory values using 12 ILIS that were available at our laboratory. Those compounds which ILIS were available were quantified using their own labelled analyte. Under these circumstances, all showed satisfactory values, indicating that both the SPE step and/or matrix effects correction was appropriate. For the rest of analytes, the selection of an analogue ILIS to correct for unsatisfactory values was rather problematic because the results might considerably vary from one sample to other. The main criterion for selection of ILIS was based on retention time similarity between the analyte and the ILIS selected, because it is expected that both will be affected by similar co-extracted constituents of the matrix. However, the use of an ILIS eluting at close retention time did not always ensure adequate correction. For example, although tylosin, erithromycin, clarithromycin and omeprazole-d_3 had similar retention times, the correction with this ILIS was unsatisfactory; however, using sulfamethoxazole-$^{13}C_6$, recoveries increased above 80% and acceptable RSD values (around 20%) were obtained. Nevertheless, for roxithromycin, although belonging to the macrolide antibiotics group and presenting similar retention time, recoveries and RSD were satisfactory making the correction with ILIS unnecessary. Another example is venlafaxine where undesirable enhancement was observed that could not be corrected with any of the available ILIS. For sulfadiazine, using acetaminophen-d_4 (the ILIS at the nearest retention time) process efficiency increased from around 15% up to around 50%. Although this approach did not fully compensate process efficiency values, it allowed improving quantitation. Despite our efforts to correct unsatisfactory data, three more compounds still presented recoveries below 60%

(norfloxacin 40%, ketoprofen 55%, naproxen 55%). For negatively ionized compounds satisfactory recoveries were normally obtained using diclofenac-d_4 as ILIS.

Based on these results, method validation in EWW was carried out, using ILIS in the way shown in Table 2. Several pharmaceuticals were quantified without ILIS correction, 12 compounds were corrected with their own ILIS, and the rest using an "analogue" ILIS. "Blank" EWW samples were spiked at two different concentration levels (100 and 500 ng/L) in quintuplicate. At the lowest level tested, marbofloxacin and paroxetine could not be validated due the poor sensitivity, and 4-aminoantipyrine, moxifloxacin and ibuprofen were not validated due to the high concentrations found in the "blank" sample. As Table 3 shows, recoveries were satisfactory (between 70% and 120%) at the two spiking levels with some exceptions. For sarafloxacin, despite using its own ILIS, the recovery was slightly lower than expected, which is in the line of our previous experiments on process efficiency in EWW (see Table 2). It seems that by any unknown reason, this ILIS did not properly correct its own analyte. In some cases, sporadic unexpected values were observed when an analogue ILIS was used (e.g. risperidone and flumequine). Nevertheless, as the RSDs were satisfactory, the use of analogues ILIS was preferred in both cases. In agreement with the previous study of matrix effects, the recoveries were non satisfactory for venlafaxine (higher than 200%) and norfloxacin (around 35%). Ketoprofen and naproxen presented normally recoveries below 70% although slightly better than expected from the study of matrix effects.

For most pharmaceuticals, the method presented satisfactory precision with RSD values even below 15% in the two fortification levels. Regarding the LOQs, they were ≤10 ng/L for 20 out of 47 compounds. For another 20 analytes they were lower than 50 ng/L. For the remaining 7 compounds, the LOQs ranged from 79 to 170 ng/L (see Table 3).

Regarding the analysis of SW, with very few exceptions, we did not observe severe matrix effects on the samples tested. However, as 12 ILIS were available, we decided to use them for correction of their own analytes and to compensate for potential errors that might occur along sample treatment and/or unexpected matrix effects. The rest of pharmaceuticals were quantified without using ILIS with the exception of 4-aminoantipyrine and risperidone that were corrected using an analogue ILIS (see Table 4). The method was tested at three fortification levels. At the lowest concentration (25 ng/L), eight compounds could not be validated due to the low sensitivity or, as for ketoprofen, due to the high analyte concentration found in the "blank" SW sample. Recoveries and RSD were mostly satisfactory at the three levels assayed (25, 100

Table 3
Method validation in effluent wastewater (EWW). Recovery (%) before and after correction with ILIS and relative standard deviation (RSD %) for five replicates.

Compound	100 ng/L		500 ng/L		LOQ (ng/L)
	Before correction	After correction	Before correction	After correction	
Acetaminophen[a]	38 (6)	120 (7)	33 (4)	104 (7)	88
Sulfadiazine	27 (10)	80 (10)	23 (2)	65 (7)	45
Furaltadone	42 (3)	74 (2)	46 (0)	65 (2)	5
Sulfathiazole	95 (3)	-	99 (3)	-	9
Pipedimic acid	32 (1)	129 (2)	18 (2)	71 (8)	91
Marbofloxacin	[b]	-	48 (6)	81 (7)	110
Trimethoprim[a]	55 (10)	86 (7)	63 (5)	85 (4)	9
Lincomycin	90 (1)	-	96 (2)	-	2
Olanzapine[a]	68 (4)	72 (8)	89 (7)	102 (12)	48
Ofloxacin	60 (15)	147 (1)	45 (6)	92 (7)	13
Pefloxacin	112 (2)	-	121 (5)	-	50
Norfloxacin	35 (5)	-	34 (4)	-	25
Furazolidone	40 (1)	75 (5)	41 (6)	83 (1)	23
Ciprofloxacin	67 (3)	147 (1)	42 (6)	92 (7)	46[d]
Enrofloxacin	116 (3)	88 (5)	120 (6)	100 (10)	21
4-Aminoantipyrine	[c]	-	81 (4)	-	23[d]
Sulfamethazine	91 (3)	-	92 (5)	-	0.8
Sulfamethoxazole[a]	59 (2)	106 (2)	46 (8)	90 (6)	13[d]
Sarafloxacin[a]	49 (8)	59 (7)	42 (11)	49 (13)	25
Salicylic acid[a]	18 (18)	92 (15)	22 (12)	101 (6)	79
Moxifloxacin	[c]	-	108 (5)	-	114
Chloramphenicol	73 (15)	-	75 (9)	-	19
Risperidone	160 (0)	150 (3)	141 (3)	127 (4)	3
Venlafaxine	230 (3)	-	218 (4)	-	7
Clindamycin	94 (2)	-	91 (1)	-	6
Enalapril	97 (7)	-	79 (3)	-	6
Paroxetine[a]	[b]	-	82 (14)	89 (9)	170
Nalidixic acid	73 (1)	-	72 (2)	-	6
Oxolinic acid	32 (3)	70 (6)	38 (4)	85 (5)	10
Flumequine	24 (7)	53 (9)	25 (2)	60 (8)	9
Omeprazole[a]	253 (3)	108 (7)	238 (3)	114 (9)	18
Tylosin	49 (8)	86 (13)	43 (5)	86 (3)	2
Erithromycin	60 (4)	108 (8)	51 (5)	104 (3)	8
Pantoprazole	111 (5)	-	100 (1)	-	4
Pravastatin	37 (14)	66 (20)	42 (7)	77 (4)	33[d]
Clarithromycin	62 (4)	125 (3)	42 (4)	85 (6)	3
Roxithromycin	59 (4)	-	59 (4)	-	17
Ketoprofen	18 (4)	48 (12)	36 (3)	70 (6)	51[d]
Lorazepam	115 (5)	-	107 (2)	-	46[d]
Alprazolam	54 (4)	-	56 (2)	-	4
Naproxen	46 (4)	85 (6)	35 (9)	66 (8)	20[d]
Bezafibrate	59 (2)	101 (6)	57 (4)	106 (7)	10[d]
Atorvastatin[a]	56 (5)	115 (2)	53 (5)	110 (5)	4[d]
Diclofenac[a]	67 (8)	106 (10)	48 (2)	97 (4)	49[d]
Ibuprofen[a]	[c]	-	71 (18)	122 (16)	150[d]
Gemfibrozil	43 (2)	101 (7)	54 (3)	105 (2)	4
Simvastatin[a]	42 (4)	109 (12)	38 (4)	98 (17)	24

Correction with ILIS made as shown in Table 2.
[a] Correction made with the analyte-labelled IS
[b] Not estimated due to the poor sensitivity.
[c] Not estimated due to the high analyte levels found in the "blank" sample.
[d] LOQ determined from the "blank" sample chromatogram (non-spiked).

Table 4
Method validation in surface water (SW). Recovery (%) before and after correction with ILIS and relative standard deviation (RSD %) for five replicates.

Compound	25 ng/L		100 ng/L		500 ng/L		LOQ (ng/L)
	Before correction	After correction	Before correction	After correction	Before correction	After correction	
Acetaminophen[a]	51 (3)	112 (1)	52 (8)	108 (8)	52 (3)	103 (2)	23
Sulfadiazine	53 (15)	-	59 (10)	-	49 (2)	-	4
Furaltadone	97 (9)	-	109 (3)	-	105 (5)	-	3
Sulfathiazole	99 (5)	-	101 (3)	-	101 (3)	-	2
Pipedimic acid	[d]	-	76 (6)	-	65 (5)	-	36
Marbofloxacin	66 (20)	-	71 (14)	-	101 (10)	-	19
Trimethoprim[a]	125 (4)	100 (3)	120 (5)	95 (3)	115 (1)	93 (2)	2
Lincomycin	72 (7)	-	90 (2)	-	94 (6)	-	2
Olanzapine[a]	73 (2)	95 (18)	119 (8)	103 (19)	76 (11)	98 (8)	9
Ofloxacin	66 (15)	-	73 (21)	-	108 (4)	-	2
Pefloxacin	108 (4)	-	116 (10)	-	120 (4)	-	13
Norfloxacin	109 (12)	-	97 (2)	-	117 (4)	-	11
Furazolidone	91 (9)	-	104 (5)	-	102 (7)	-	1
Ciprofloxacin	70 (6)	-	82 (5)	-	101 (1)	-	18
Enrofloxacin	120 (10)	-	117 (10)	-	156 (4)	-	9
4-Aminoantipyrine[b]	54 (4)	97 (11)	52 (11)	81 (22)	50 (5)	94 (9)	1
Sulfamethazine	109 (13)	-	123 (3)	-	109 (15)	-	0.5
Sulfamethoxazole[a]	106 (3)	108 (3)	104 (2)	94 (6)	99 (3)	99 (3)	3
Sarafloxacin[a]	74 (5)	77 (13)	63 (14)	66 (11)	71 (7)	73 (3)	10
Salicylic acid[a]	[d]	-	38 (18)	102 (15)	42 (16)	105 (13)	76
Moxifloxacin	[d]	-	121 (10)	-	149 (4)	-	55
Chloramphenicol	102 (11)	-	97 (1)	-	109 (4)	-	7
Risperidone[c]	139 (2)	118 (3)	128 (4)	122 (9)	137 (3)	115 (5)	2
Venlafaxine	133 (3)	-	135 (4)	-	142 (4)	-	3
Clindamycin	72 (14)	-	83 (1)	-	90 (7)	-	1
Enalapril	78 (4)	-	83 (3)	-	76 (4)	-	4
Paroxetine[a]	[d]	-	108 (11)	86 (6)	118 (12)	91 (4)	29
Nalidixic acid	86 (4)	-	95 (5)	-	101 (8)	-	3
Oxolinic acid	70 (5)	-	81 (2)	-	80 (9)	-	2
Flumequine	72 (15)	-	77 (9)	-	76 (12)	-	2
Omeprazole[a]	196 (5)	115 (13)	159 (2)	107 (3)	134 (10)	109 (3)	2
Tylosin	51 (6)	-	52 (6)	-	54 (5)	-	0.7
Erithromycin	63 (9)	-	67 (9)	-	73 (8)	-	0.4
Pantoprazole	118 (3)	-	115 (4)	-	114 (5)	-	2
Pravastatin	[d]	-	104 (8)	-	89 (2)	-	23
Clarithromycin	72 (14)	-	72 (11)	-	82 (6)	-	2
Roxithromycin	69 (8)	-	74 (10)	-	84 (16)	-	2
Ketoprofen	[e]	-	92 (14)	-	99 (10)	-	29
Lorazepam	111 (13)	-	118 (4)	-	99 (0)	-	8
Alprazolam	111 (6)	-	107 (2)	-	109 (1)	-	1
Naproxen	85 (17)	-	91 (8)	-	81 (3)	-	20
Bezafibrate	106 (7)	-	100 (0)	-	102 (2)	-	3
Atorvastatin[a]	14 (21)	91 (8)	21 (10)	103 (3)	21 (18)	97 (3)	3
Diclofenac[a]	116 (15)	103 (11)	112 (5)	99 (8)	104 (2)	103 (3)	4
Ibuprofen[a]	[d]	-	92 (16)	110 (15)	89 (21)	105 (17)	85
Gemfibrozil[a]	105 (12)	-	103 (8)	-	97 (1)	-	9
Simvastatin[a]	[d]	-	30 (8)	102 (5)	34 (10)	106 (9)	18

[a] Correction made with the analyte-labelled IS.
[b] Correction made with acetaminophen-d_4.
[c] Correction made with sarafloxacin-d_8.
[d] Not estimated due to the poor sensitivity.
[e] Not estimated due to the high analyte levels found in the "blank" sample.

and 500 ng/L). Only two compounds (moxifloxacin and venlafaxine) showed values higher than 120% and another two (sulfadiazine and tylosin) yielded recoveries around 50%. The LOQs in SW were lower than 20 ng/L for the majority of compounds (39 out of 47). Similarly to EWW, the LOQs for salicylic acid, moxifloxacin and ibuprofen were among the highest, as a consequence of the poor sensitivity of the method for these compounds.

3.4. Application to environmental water samples

It is important to remark that matrix effects are a problematic issue in environmental analysis, particularly when dealing with multi-residue methods. This problem is not easy to solve, as discussed in the previous section and reported in the bibliography. The unavailability of true blank samples to perform a calibration in matrix, and the extreme difficulties to get the ILIS required for every analyte in a method for a large number of compounds, make necessary to find a realistic compromise between time, analytical efforts and amount and quality of information obtained. The composition of environmental water or wastewater is never the same. So, unexpected matrix effects could occur in every analysis, even if the method has been satisfactorily tested in similar matrices. Although the use of analogues ILIS can be a satisfactory solution in some particular cases, this approach does not ensure an appropriate correction in all samples analyzed as reported in this work or in previous articles [29,31,32]. In the absence of the own analyte ILIS, the most reliable option seems to be the application of the standard additions methods, or to perform an extensive clean-up of the extracts. However, both approaches are time-consuming and can increase the analytical errors due to the sample manipulation. The standard additions method, typically reported as one of the best ways to get accurate data, is not so easy to apply when analyzing ng/L levels. To obtain satisfactory data, it requires a previous analysis to have an estimation of the concentration level in sample in order to adjust the concentrations added. Then, it is necessary to have extreme care when obtaining the calibration with each sample, as calculating the concentration by extrapolation can lead to very high errors. Finally, the number of samples analyzed increases by a factor of 4–5, i.e. the points corresponding to the different additions made. By other side, to ensure an efficient clean-up when sample composition is highly variable and when a method is applied for a large number of compounds is rather complicated, as the method would end up being more restrictive, which is the opposite to that pursued in multi-residue methods.

Table 5
Summary of the results obtained for target pharmaceuticals.

Compound	Surface water (n = 18)		Effluent wastewater (n = 19)	
Compound	% positive findings	Maximum level (ng/L)	% positive findings	Maximum level (ng/L)
Acetaminophen	72	1968	21	201
4-Aminoantipyrine	50	811	84	2770
Alprazolam	0	-	47	7
Atorvastatin	11	42	74	209
Bezafibrate	39	49	79	312
Chloramphenicol	0	-	0	-
Ciprofloxacin	100	740	100	2292
Clarithromycin	56	91	74	247
Clindamycin	0	-	0	-
Diclofenac	61	358	84	690
Enalapril	44	88	16	236
Enrofloxacin	100	70	53	220
Erithromycin	44	78	74	82
Flumequine	83	20	11	41
Furaltadone	0	-	5	9
Furazolidone	0	-	0	-
Gemfibrozil	28	304	84	2008
Ibuprofen	22	2850	21	15,1
Ketoprofen	44	70	79	583
Lincomycin	50	47	79	142
Lorazepam	0	-	79	81
Marbofloxacin	0	-	0	-
Moxifloxacin	17	205	16	540
Nalidixic acid	39	14	6	60
Naproxen	44	285	79	710
Norfloxacin	100	54	89	310
Ofloxacin	100	400	100	925
Olanzapine	39	58	21	< LOQ
Omeprazole	0	-	32	30
Oxolinic acid	83	23	0	-
Pantoprazole	50	117	47	36
Paroxetine	0	-	0	-
Pefloxacin	22	64	5	112
Pipedimic acid	11	245	68	430
Pravastatin	0	-	16	69
Risperidone	0	-	0	-
Roxithromycin	11	12	42	18
Salicylic acid	61	1160	74	80
Sarafloxacin	89	55	16	52
Simvastatin	0	-	0	-
Sulfadiazine	0	-	0	-
Sulfamethazine	0	-	5	11
Sulfamethoxazole	28	33	84	432
Sulfathiazole	0	-	11	30
Trimethoprim	28	151	84	232
Tylosin	0	-	0	-
Venlafaxine	39	575	74	875

Taking into account all previous considerations, quantitative data presented in this work should possibly be taken as estimated levels, with the exception of those analytes that are corrected with their own ILIS. Despite that QCs included in every sequence of samples analyzed were satisfactory, from a strict point of view no fully correction would be ensured in those cases where the own analyte ILIS could not be used. In the case that the concentrations reported had severe implications (levels above the maximum allowed in the legislation), which seems not to be the case with emerging contaminants that are still unregulated in water, a highly reliable quantification would be required in a second analysis.

The method developed was applied to 18 SW collected from different sampling points in Mediterranean rivers (Valencia region) and to 19 EWW from different WWTPs from this region (see Table 5).

In every sequence of analysis, the calibration curve was injected twice, at the beginning and the end of the sample batch. Moreover, quality control samples (QCs) were included in every sequence in order to assure the quality of the analysis. QCs consisted on SW or EWW that were spiked to 100 ng/L with all pharmaceuticals. They were analyzed following the same analytical procedure than the samples. QC recoveries in the range 70–120% were considered as satisfactory.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q) transition, and comparing them with the ion-ratio calculated from a reference standard. The finding was considered as true positive when the experimental ion-ratio was within the tolerance range [26] and the retention time of the compound in the sample within ±2.5% the retention time of the reference standard. Thus, the method fulfills the European Union guidelines and it ensures accurate identification of target analytes [26].

In SW, up to 31 pharmaceuticals were detected at least once. Analgesic and anti-inflammatories, cholesterol lowering statin drugs and lipid regulators, and quinolone antibiotics were the most detected groups. The highest concentrations corresponded to ibuprofen, acetaminophen and salicylic acid with maximum levels of 2.9, 1.9, and 1.2 μg/L, respectively. These compounds are included in the list of the 35 most consumed active principles with medical prescription in Spain [19], although they may also be acquired without medical

prescription. These occurrences are in accordance with studies of other countries where a similar situation has been observed [21].

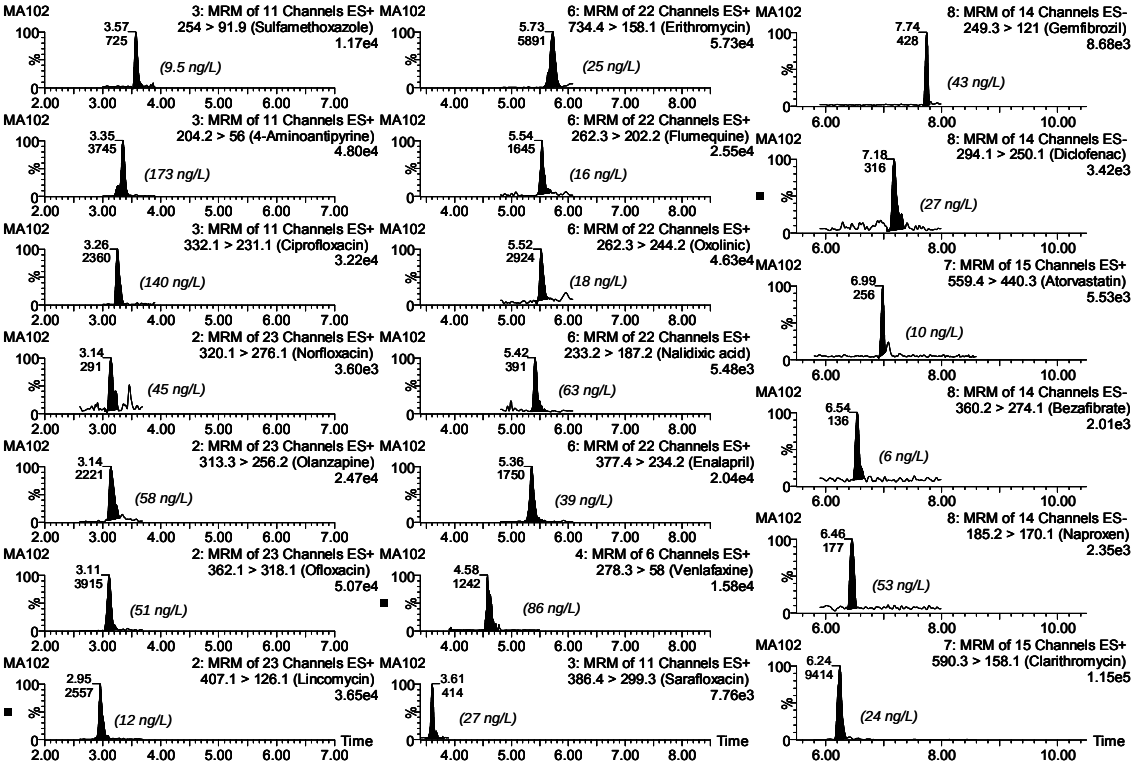

Figure 1. UHPLC-MS/MS chromatograms (Q transition) for a surface water that was positive to 20 pharmaceuticals. ES+ and ES- was simultaneously applied within the same run.

Regarding EWW, a higher number of target compounds were detected at least once (37 out of 47). Some of them, as ciprofloxacin and ofloxacin, were detected in 100% of the samples. Compounds belonging to the cholesterol lowering statin drugs and lipid regulators group were also frequently detected, except for simvastatin which was not found in either SW or EWW. This compound is the fourth most consumed in Spain [19] and its absence might be explained by the transformation of the parent compound in the aquatic environment.

Omeprazole and lorazepam were not detected in SW; however, they were present in around 30% and 80% of the EWW samples, respectively. A similar situation was observed for trimethoprim and atorvastatin that were hardly found in SW, but were detected in around 80% of the EWW samples. This fact might be due to the dilution of pharmaceuticals when they reach surface water together with transformation processes. Similarly to SW, the highest concentrations in EWW were found for acetaminophen (around 200 μg/L), salicylic acid (80 μg/L) and ibuprofen (15 μg/L), which were notably higher than in SW possibly due to the dilution process suffered in SW. Quantification of the EWW samples with high analyte levels required an additional analysis previous dilution of the sample before the SPE step.

The majority of the EWW samples analyzed were positive for at least 20 out of 47 target compounds. We did not expect to find so many positives in SW as we presumed they were less affected by the presence of pharmaceuticals. However, around 50% of the SW samples contained at least 19 analytes. These data reinforce the need of applying multi-class methods to obtain a wider and realistic knowledge on the occurrence of pharmaceuticals in environmental water. As an illustrative example, Fig. 1 shows UHPLC–MS/MS chromatograms for a SW sample which was positive to 20 compounds, with concentrations varying from 6 ng/L (bezafibrate) to 173 ng/L (4-aminoantipyrine). The high sensitivity of the method allowed us the detection and confirmation of analytes at concentrations around, or even below, the LOQ level, as shown in Fig. 2 for a EWW sample.

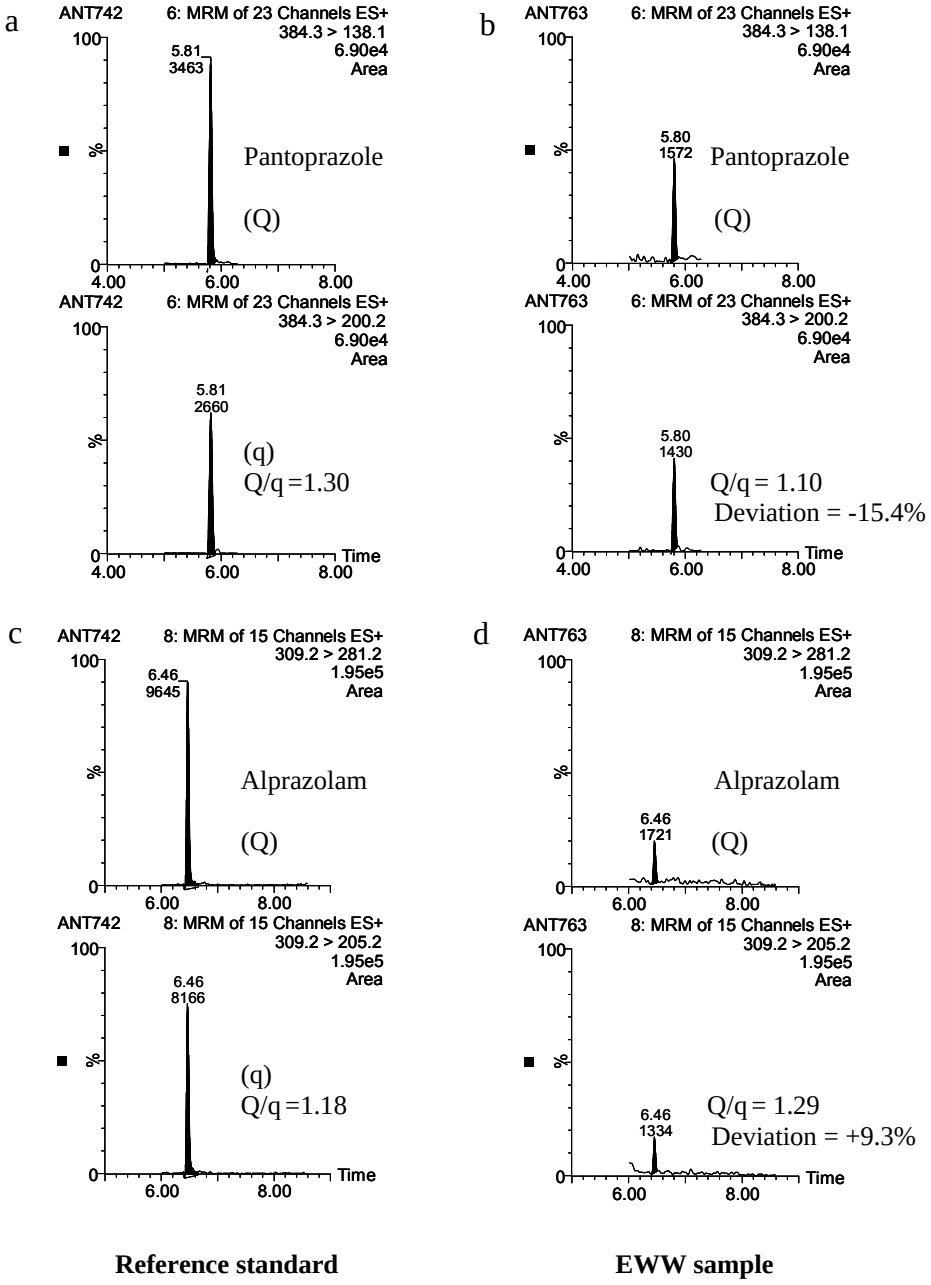

Figure 2. Selected UHPLC-MS/MS chromatograms for (a) pantoprazole reference standard (1 µg/L), (b) effluent wastewater sample containing 0.005 µg/L of pantoprazole, (c) alprazolam reference standard (1 µg/L) and (d) effluent wastewater sample (estimated concentration 0.002 µg/L of alprazolam).

4. Conclusions

In this paper, advanced UHPLC–MS/MS analytical methodology has been developed for the simultaneous quantification and confirmation of 47 pharmaceuticals in surface water and wastewater samples. The proposed methodology allows the extraction of all pharmaceuticals in a single SPE step and their simultaneous determination under positive and negative electrospray modes with a chromatographic run of only 10 min. Two SRM transitions have been acquired per compound for a reliable identification.

Special attention has been paid to the correct quantification of analytes, which is more problematic in wastewater due to the presence of co-extracted components of the sample that can produce severe matrix effects. The use of 12 ILIS has been tested to correct undesirable effects for the 47 selected compounds in nine EWW, collected from different WWTPs. Appropriate correction was ensured in all samples tested only when ILIS were used to correct their own analyte. Also a correction with analogue ILIS, different from the labelled analyte, was required for several compounds in EWW. However, matrix variability of environmental water and waste water makes this correction problematic. Therefore, the evaluation and correction of matrix effects should not be based on the behaviour of an analogue ILIS in only a few samples (e.g. one or two samples), but in several random samples that represent the matrix variability along the time. In addition, quality control samples should always be included in every sequence of analysis to test if the analogue ILIS leads to confident quantitative data actually.

The high number of target compounds and the rather different class of therapeutical groups made of this method one of the most advanced in relation to its wide scope. In addition, pharmaceuticals have been selected based on their wide consumption and/or potential negative effects (mainly antibiotics). Therefore, considering that positively and negatively ionized compounds are simultaneously determined in just one injection, this method can offer a more realistic overview of the water quality as regards pharmaceuticals contamination than most methods previously reported. The interest of increasing the number of analytes is clearly shown by the fact that almost all compounds selected have been detected in the water samples analyzed.

In the near future, the presence of metabolites will be investigated by using a quadrupole time-of-flight mass analyzer. This investigation might be on interest especially for those compounds that are not detected in water despite their frequent use, e.g. simvastatin.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.026.

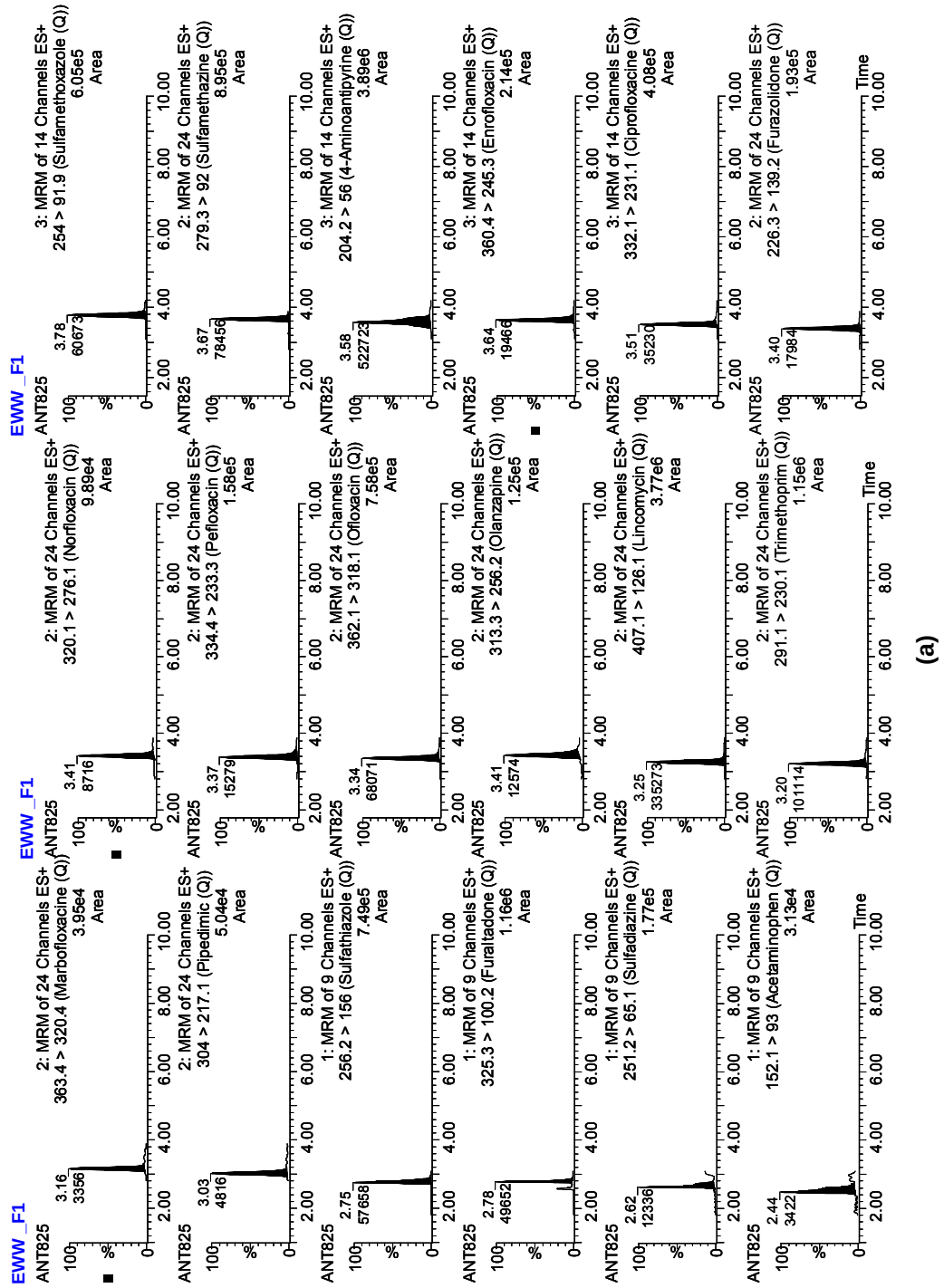
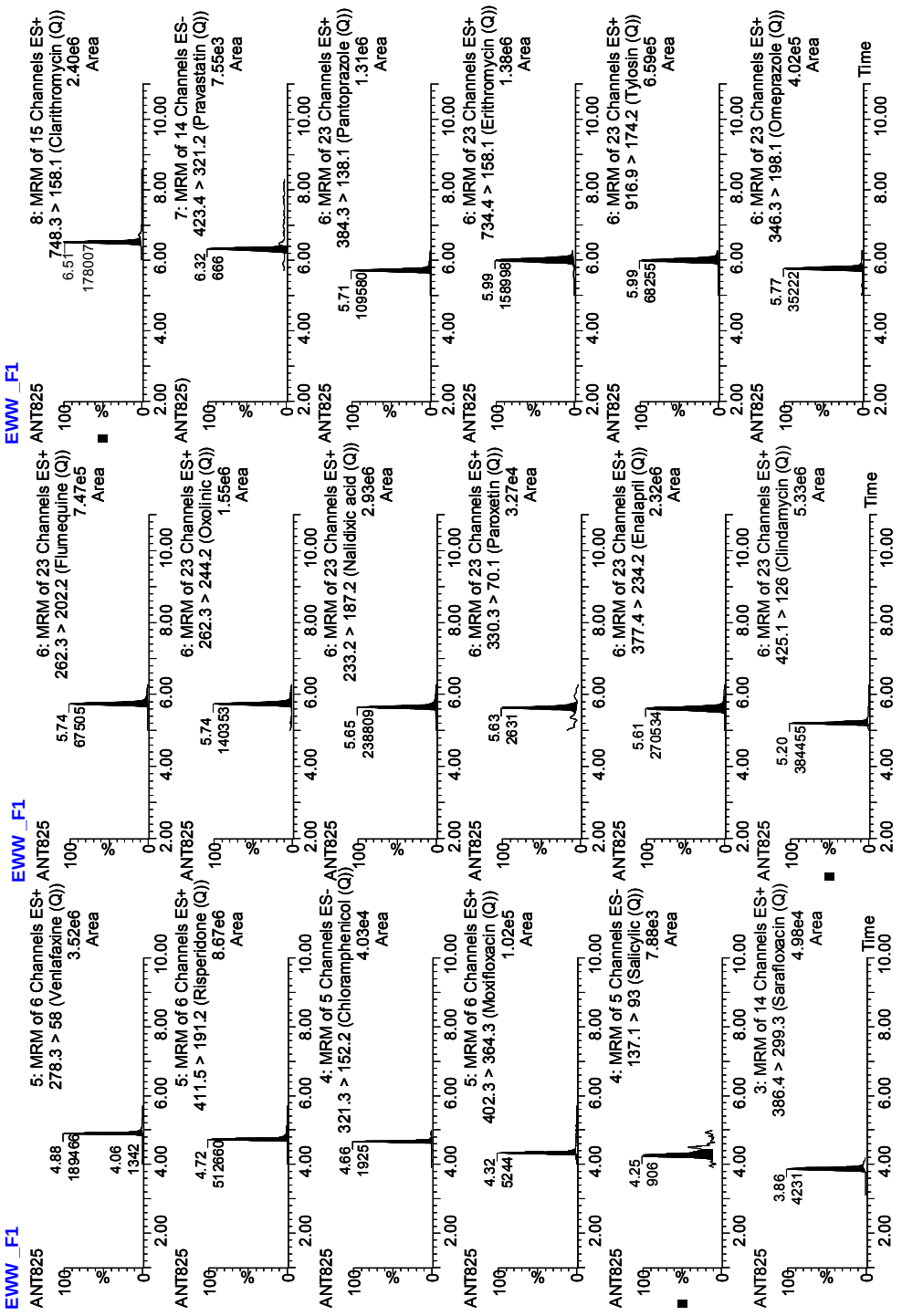
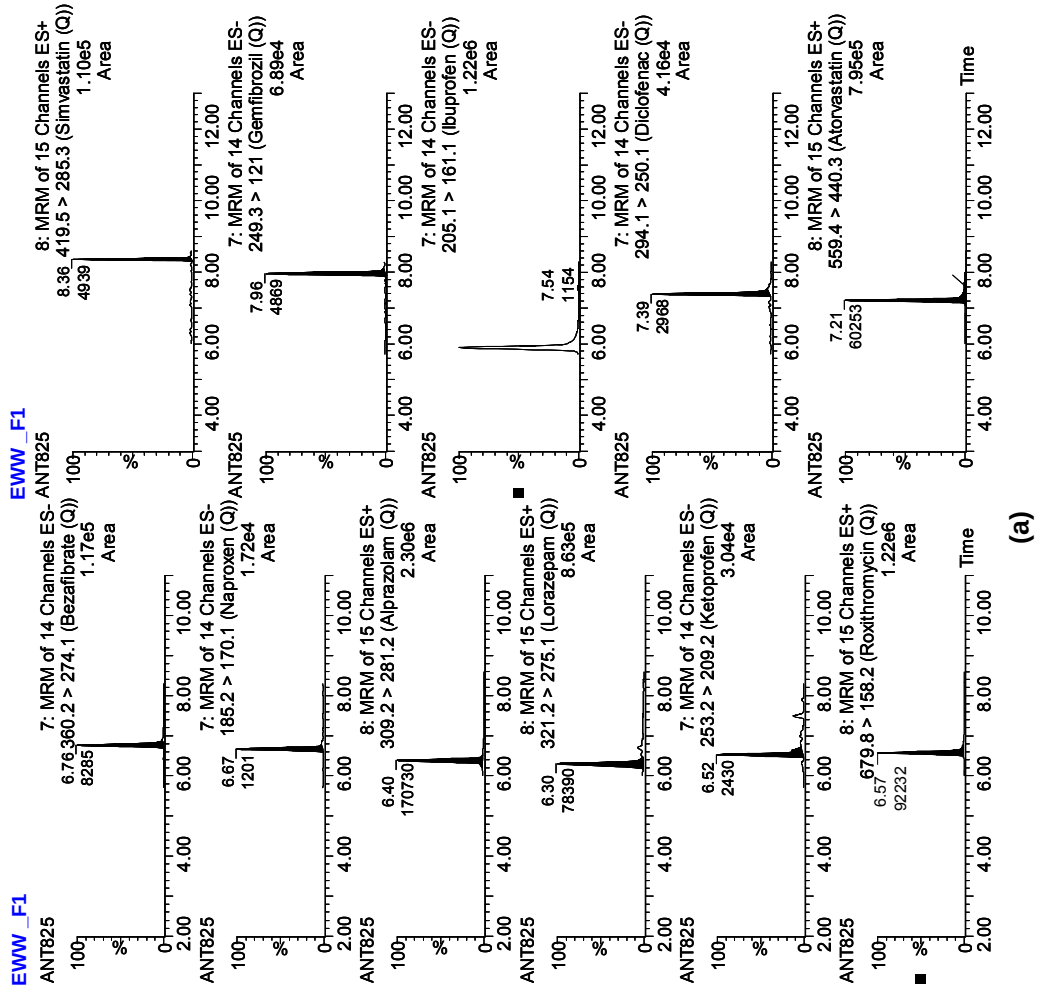
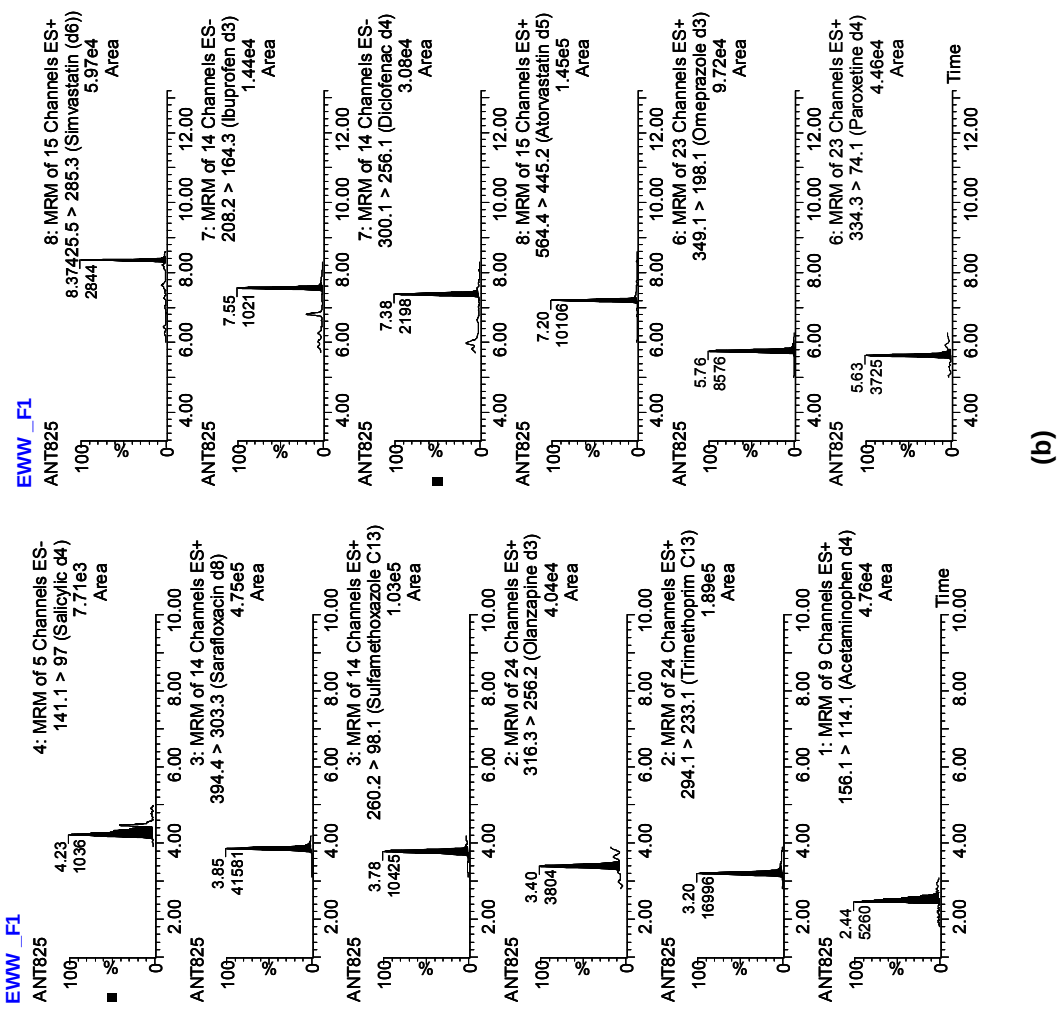


Figure 1. UHPLC-MS/MS chromatograms (Q transition) for EWW spiked at 0.5 µg/L (a) pharmaceuticals, (b) ILIS


EWW_F1
ANT825
5: MRM of 6 Channels ES+
278.3 > 58 (Venlafaxine (Q))
3.52e6
Area
4.88
189466
4.06
1342
6: MRM of 23 Channels ES+
262.3 > 202.2 (Flumequine (Q))
7.47e5
Area
5.74
67505
8: MRM of 15 Channels ES+
748.3 > 158.1 (Clarithromycin (Q))
2.40e6
Area
6.51
178007
5: MRM of 6 Channels ES+
411.5 > 191.2 (Risperidone (Q))
8.67e6
Area
4.72
512660
6: MRM of 23 Channels ES+
262.3 > 244.2 (Oxolinic (Q))
1.55e6
Area
5.74
140353
7: MRM of 14 Channels ES-
423.4 > 321.2 (Pravastatin (Q))
7.55e3
Area
6.32
666
4: MRM of 5 Channels ES-
321.3 > 152.2 (Chloramphenicol (Q))
4.03e4
Area
4.66
1925
6: MRM of 23 Channels ES+
233.2 > 187.2 (Nalidixic acid (Q))
2.93e6
Area
5.65
238809
6: MRM of 23 Channels ES+
384.3 > 138.1 (Pantoprazole (Q))
1.31e6
Area
5.71
109580
5: MRM of 6 Channels ES+
402.3 > 364.3 (Moxifloxacin (Q))
1.02e5
Area
4.32
5244
6: MRM of 23 Channels ES+
330.3 > 70.1 (Paroxetin (Q))
3.27e4
Area
5.63
2631
6: MRM of 23 Channels ES+
734.4 > 158.1 (Erithromycin (Q))
1.38e6
Area
5.99
158998
4: MRM of 5 Channels ES-
137.1 > 93 (Salicylic (Q))
7.88e3
Area
4.25
906
6: MRM of 23 Channels ES+
377.4 > 234.2 (Enalapril (Q))
2.32e6
Area
5.61
270534
6: MRM of 23 Channels ES+
916.9 > 174.2 (Tylosin (Q))
6.59e5
Area
5.99
68255
3: MRM of 14 Channels ES+
386.4 > 299.3 (Sarafloxacin (Q))
4.98e4
Area
3.86
4231
6: MRM of 23 Channels ES+
425.1 > 126 (Clindamycin (Q))
5.33e6
Area
5.20
384455
6: MRM of 23 Channels ES+
346.3 > 198.1 (Omeprazole (Q))
4.02e5
Area
5.77
35222
Time
2.00
4.00
6.00
8.00
10.00
%
0
100

(a)


EWW_F1
ANT825
7: MRM of 14 Channels ES-
360.2 > 274.1 (Bezafibrate (Q))
6.76
8285
1.17e5
Area
8: MRM of 15 Channels ES+
419.5 > 285.3 (Simvastatin (Q))
8.36
4939
1.10e5
Area
7: MRM of 14 Channels ES-
185.2 > 170.1 (Naproxen (Q))
6.67
1201
1.72e4
Area
7: MRM of 14 Channels ES-
249.3 > 121 (Gemfibrozil (Q))
7.96
4869
6.89e4
Area
8: MRM of 15 Channels ES+
309.2 > 281.2 (Alprazolam (Q))
6.40
170730
2.30e6
Area
7: MRM of 14 Channels ES-
205.1 > 161.1 (Ibuprofen (Q))
7.54
1154
1.22e6
Area
8: MRM of 15 Channels ES+
321.2 > 275.1 (Lorazepam (Q))
6.30
78390
8.63e5
Area
7: MRM of 14 Channels ES-
294.1 > 250.1 (Diclofenac (Q))
7.39
2968
4.16e4
Area
7: MRM of 14 Channels ES-
253.2 > 209.2 (Ketoprofen (Q))
6.52
2430
3.04e4
Area
8: MRM of 15 Channels ES+
559.4 > 440.3 (Atorvastatin (Q))
7.21
60253
7.95e5
Area
8: MRM of 15 Channels ES+
679.8 > 158.2 (Roxithromycin (Q))
6.57
92232
1.22e6
Area
Time
(a)


EWW_F1
ANT825
4: MRM of 5 Channels ES-
141.1 > 97 (Salicylic d4)
7.71e3
Area
4.23
1036
3: MRM of 14 Channels ES+
394.4 > 303.3 (Sarafloxacin d8)
4.75e5
3.85
41581
3: MRM of 14 Channels ES+
260.2 > 98.1 (Sulfamethoxazole C13)
1.03e5
3.78
10425
2: MRM of 24 Channels ES+
316.3 > 256.2 (Olanzapine d3)
4.04e4
3.40
3804
2: MRM of 24 Channels ES+
294.1 > 233.1 (Trimethoprim C13)
1.89e5
3.20
16996
1: MRM of 9 Channels ES+
156.1 > 114.1 (Acetaminophen d4)
4.76e4
2.44
5260
8: MRM of 15 Channels ES+
425.5 > 285.3 (Simvastatin (d6))
5.97e4
8.37
2844
7: MRM of 14 Channels ES-
208.2 > 164.3 (Ibuprofen d3)
1.44e4
7.55
1021
7: MRM of 14 Channels ES-
300.1 > 256.1 (Diclofenac d4)
3.08e4
7.38
2198
8: MRM of 15 Channels ES+
564.4 > 445.2 (Atorvastatin d5)
1.45e5
7.20
10106
6: MRM of 23 Channels ES+
349.1 > 198.1 (Omeprazole d3)
9.72e4
5.76
8576
6: MRM of 23 Channels ES+
334.3 > 74.1 (Paroxetine d4)
4.46e4
5.63
3725
Area
%
Time
(b)

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2.3.3 Discusión de los resultados (artículo científico 2)

Optimización de las condiciones MS/MS

La mayor parte de los compuestos se determinaron en modo de ionización positivo y tan sólo una nueve analitos presentaron ionización en modo negativo. Para todos los compuestos se seleccionó como ion precursor $[M+H]^+$ o $[M-H]^-$, excepto para naproxeno, roxitromicina y claritromicina. En estos casos se aprovechó su facilidad de fragmentación a bajas energías para seleccionar un fragmento como ión precursor en la transición de confirmación, promoviendo su formación mediante la aplicación de un voltaje elevado. Dichos fragmentos se seleccionaron como iones precursores porque su intensidad era mayor que el ion de la molécula (des)protonada. En la Figura 2.3 se muestran los espectros MS y MS/MS de la roxitromicina y del naproxeno obtenidos mediante la infusión de un patrón de 1 ppm.

Para cada compuesto se seleccionaron dos transiciones con el fin de confirmar su presencia. Las únicas excepciones fueron el ibuprofeno, ketoprofeno y ácido salícilico para los que solamente fue posible seleccionar una transición debido a la presencia de un único ion producto. En la medida de lo posible se evitaron las transiciones poco selectivas (ej. pérdidas de agua) con el fin de aumentar la selectividad del método y disminuir la posibilidad de reportar falsos positivos.

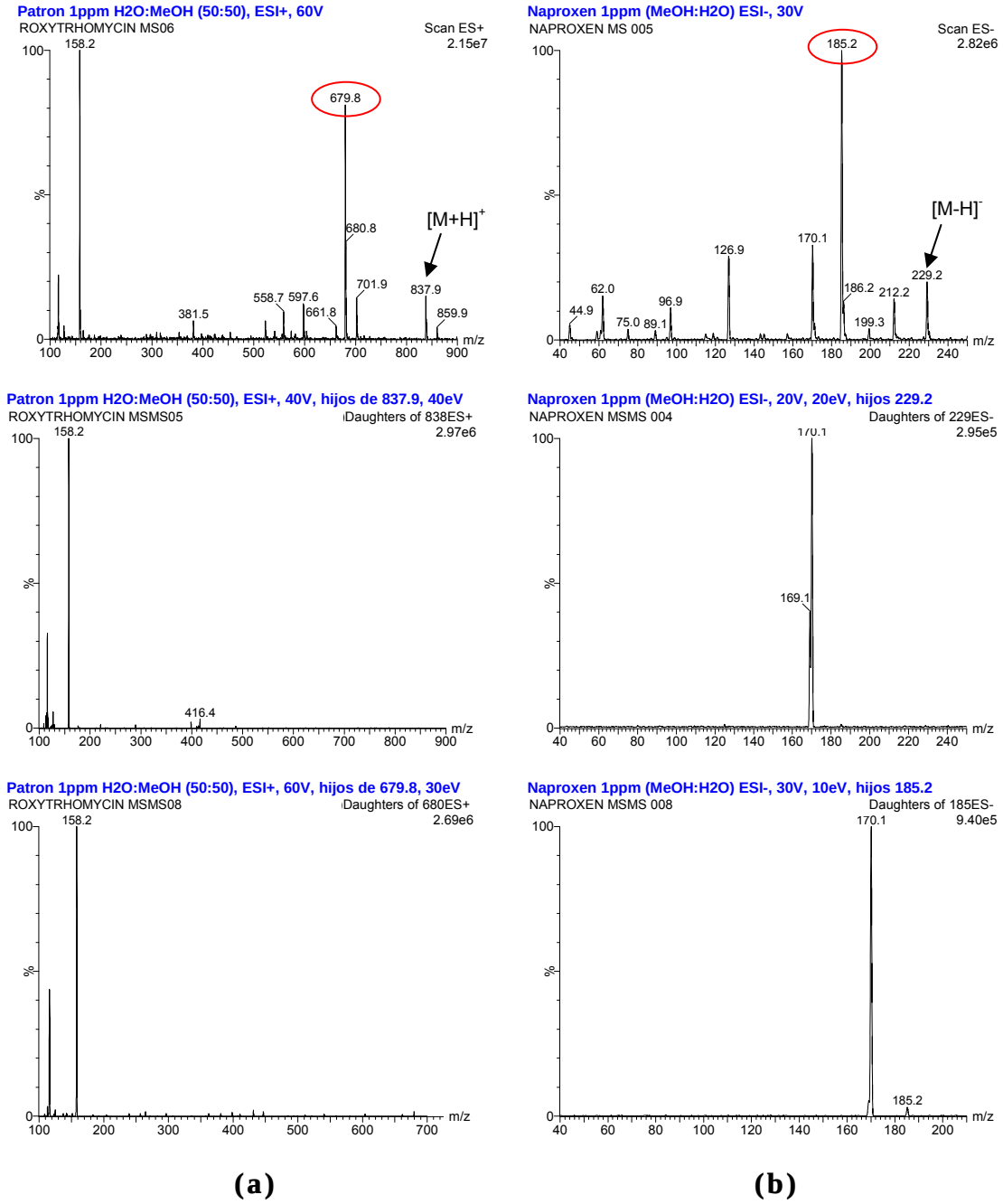

Figura 2.3 Espectros MS y MS/MS de (a) roxitromicina y (b) naproxeno

Optimización cromatográfica y etapa de extracción

Dado el elevado numero de analitos, se seleccionó una columna más larga que la utilizada en el trabajo anterior. De este modo, fue posible separarlos cromatográficamente y analizarlos en tan sólo 10 minutos.

Como se ha comentado en el Capítulo 1, cuando se trabaja con un número de compuestos tan elevado como en este trabajo resulta complicado encontrar las condiciones óptimas de sensibilidad y de forma de pico para cada uno de los compuestos. La dificultad es todavía mayor cuando se analizan simultáneamente compuestos que se ionizan en modo negativo y en modo positivo. Eso obliga a seleccionar unas condiciones de compromiso que sirvan para todos ellos. Este mismo problema se plantea a la hora de seleccionar las condiciones de la etapa de extracción.

Optimización del método

Como es bien sabido, el efecto matriz es una de las principales limitaciones de los métodos LC-MS/MS. Además, los problemas asociados al proceso de extracción SPE también pueden afectar a la eficacia y robustez del método. Por ese motivo, en este trabajo se realizó un estudio detallado de ambas variables (efecto matriz y posibles pérdidas durante el proceso de extracción), analizando su impacto en el proceso de cuantificación. Con dicho fin se determinó la eficacia global del proceso (PE) utilizando nueve aguas de efluente de distinto origen. Se estudió la posibilidad de corregir los problemas asociados mediante el uso de doce compuestos marcados isotópicamente (tres de ellos con ionización en modo negativo). Para ello se compararon las respuestas obtenidas en las muestras fortificadas antes de la etapa de SPE con la respuesta del patrón en solvente.

En la *Tabla 2, artículo científico 2* se muestran los valores PE promedio de las nueve muestras junto con el valor de RSD, antes y después de la corrección con los compuestos marcados. El valor de RSD, que permite estimar la repetibilidad de los

valores obtenidos, nos permitió estimar si el comportamiento de los analitos fue similar o diverso en las distintas aguas analizadas.

Para la mayoría de los compuestos se obtuvieron valores de PE menores de 100%. Este dato implica una supresión de la señal debido al efecto matriz y/o pérdidas durante el proceso de extracción. Tan sólo para unos pocos compuestos el valor de PE fue mayor de 100% debido probablemente a una exaltación de la señal producida por la matriz. Estos resultados indican que para poder cuantificar correctamente los analitos presentes en las muestras se requiere una corrección. De acuerdo con lo expuesto en el Capítulo 1, el uso de compuestos marcados isotópicamente es hoy en día el modo más utilizado para la corrección del efecto matriz. Sin embargo, en los métodos multirresiduales su uso presenta importantes limitaciones ya que resulta poco factible poder corregir cada analito con su propio analito marcado cuando el número de analitos es elevado. En aquellos compuestos para los que no disponíamos de su marcado optamos por agruparlos según su tiempo de retención y corregir sus respuestas con el compuesto marcado más cercano. Cabe esperar que si un analito y un compuesto marcado eluyen a un tiempo de retención muy similar ambos estarán afectados por los mismos constituyentes de la matriz. Este criterio condujo, en general, a resultados satisfactorios. Sin embargo, encontramos algunos casos en los que resultó más adecuado utilizar un compuesto marcado con un tiempo de retención más alejado en lugar del análogo más cercano. Por otro lado, dentro de una misma familia química y con tiempos de retención similares, algunos compuestos requieron corrección mientras que otros no.

Para la mayoría de los compuestos, los valores de PE mejoraron al utilizar un compuesto marcado análogo (véase la *Tabla 2*, *artículo científico 2*). Además, los valores de RSD también mejoraron, siendo en la mayoría de los casos inferiores al 20%. Este hecho parece indicar que, tras la corrección con los compuestos marcados, el comportamiento de los analitos fue similar en la mayoría de las muestras. Por ello, el método desarrollado puede considerarse reproducible. Únicamente en el caso de tres compuestos no fue posible mejorar la eficacia del proceso con ninguno de los compuestos deuterados disponibles.

Nuestros resultados ponen de manifiesto, una vez más, que el efecto matriz depende tanto de la matriz como del propio analito. Por ese motivo, resulta imposible poder predecir *a priori* el grado de efecto matriz que experimenta un compuesto y si un analito marcado análogo será capaz de corregir un compuesto o no. Por lo tanto, se requiere llevar a cabo un amplio estudio que trate de afrontar distintas situaciones posibles y composiciones de matriz. Para evaluar el efecto matriz y su posible corrección en este trabajo se utilizaron aguas procedentes de distintas depuradoras tomadas en diferentes epocas del año. Hay que tener presente que la composición de las muestras de agua nunca es la misma y, por tanto, pueden producirse efectos matriz inesperados. Por ello, cuando se utilizan compuestos marcados análogos se debería incluir al menos una muestra de control de calidad (QC) en la secuencia de análisis para comprobar que su comportamiento es el esperado.

Validación del método

El método fue validado satisfactoriamente en agua superficial a tres niveles de fortificacion (0.025, 0.1 y 0.5 µg/L) y en efluente a dos niveles (0.1 y 0.5 µg/L). En las aguas superficiales las experiencias previas indicaron que el efecto matriz experimentado por los analitos no suponía un problema para su cuantificacion. Sin embargo, se decidió utilizar los compuestos deuterados disponibles para corregir a sus propios analitos y compensar los posibles errores producidos durante el proceso y/o efectos matriz inesperados. Además, para dos compuestos (4-aminoantipirina y risperidona) los valores de recuperación mejoraron al aplicarles un analito marcado análogo.

En agua de efluente, la validación del metodo se llevó a cabo basándonos en los resultados obtenidos en el estudio previo anteriormente descrito.

En ambas matrices, y como era de esperar, los compuestos cuya molécula marcada fue usada como patrón interno fueron satisfactoriamente corregidos. Tan sólo en el caso de uno de ellos (sarafloxacino-d_8) la corrección no resultó adecuada. Una explicación posible podría ser que, como se ha comentado en el Capítulo 1, los analitos

marcados con un número elevado de isótopos deuterados pueden tener un comportamiento físico-químico distinto al del analito.

Análisis de muestras. Resultados en aguas superficiales y en efluentes urbanos

La metodología descrita se aplicó a muestras de agua superficiales y de efluentes. Las muestras superficiales se tomaron en diferentes puntos de ríos de la vertiente mediterránea. Las muestras de efluentes procedían de distintas EDAR de la Comunidad Valenciana. Cabe indicar que en cada secuencia de analisis se incluyeron muestras QCs para asegurar la calidad de los análisis.

En las aguas superficiales se encontraron numerosos compuestos estudiados. A modo de ejemplo, en la *Figura 1* del artículo se muestra un cromatograma de una muestra superficial que contenía 20 de los fármacos analizados.

En el caso de las muestras de efluente, el 80% de los analitos se detectó, al menos, en alguna ocasión. Como era de esperar, la frecuencia de detección de los compuestos así como sus niveles de concentración fueron más elevados que en las aguas superficiales. Los compuestos que presentaron mayor nivel de concentración fueron paracetamol, ibuprofeno y ácido salicílico, al igual que en las muestras de agua superficial. Estos resultados concuerdan con los del *artículo científico 1.*

En relación a los antibióticos, el grupo de los antibióticos betalactámicos -especialmente las penicilinas y cefalosporinas- es el más consumido en Europa (Kümmerer, 2009; ESAC; ECDC). En segundo lugar se encuentra el grupo de los macrólidos, seguido de las tetraciclinas, fluoroquinolonas y sulfonamidas (Moreno-Bondi, 2009). En las Figuras 2.4 y 2.5 se ha representado el consumo de los antibióticos por grupos en la Unión Europea.

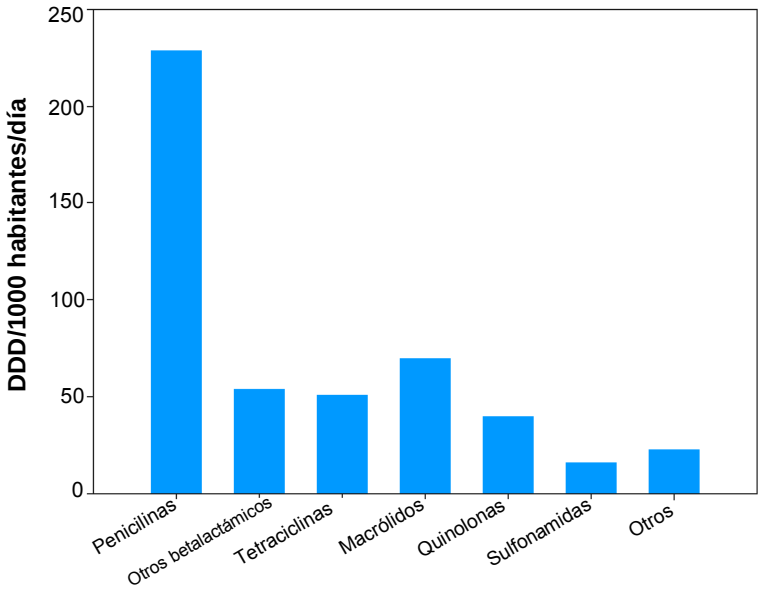

Figura 2.4 Datos sobre el consumo de antibióticos, expresado en dosis diarias definidas (DDD) por 1000 habitantes y por día en 2006 en 24 países de Europa. Modificado de Moreno-Bondi, 2009.

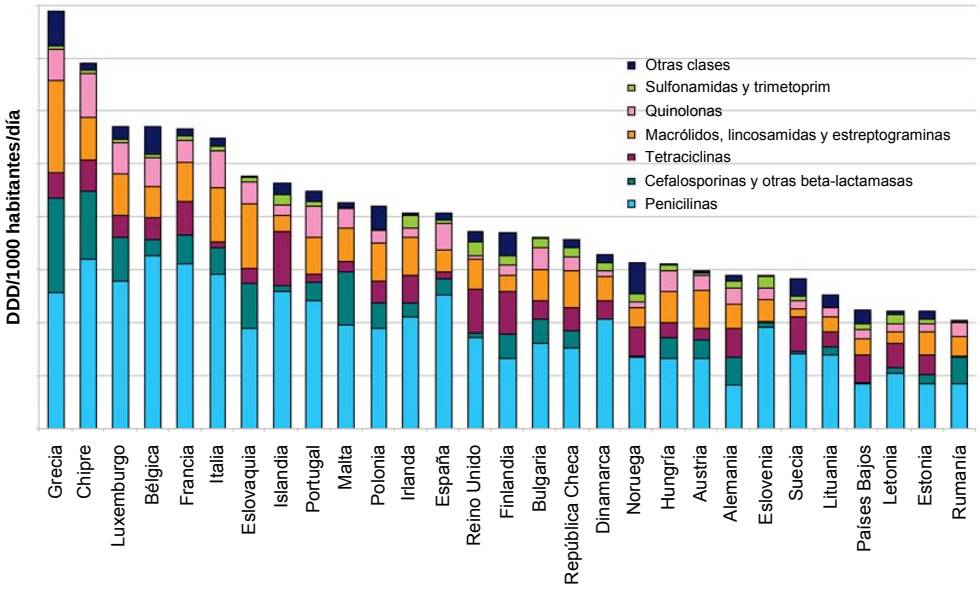

Figura 2.5 Consumo extrahospitalario de antibióticos de uso sistémico en 29 países de la Unión Europea (datos correspodientes al año 2010s suministrados por el Centro Europeo de Prevención y Control de Enfermedes, ECDC)

Los betalactámicos se hidrolizan con facilidad (Moreno-Bondi, 2009) y por ello se suelen encontrar a niveles muy bajos de concentración e inferiores al del resto de antibióticos. En cambio, las quinolonas, los macrólidos y las sulfonamidas son mucho más estables (Cha, 2006) y se detectan con frecuencia en el agua superficial y en la de efluente.

En este estudio, las concentraciones más elevadas correspondieron a ciprofloxacino, ofloxacino, ácido pipemídico y sulfametoxazol. Los tres primeros pertenecen al grupo de las quinolonas y el cuarto al de las sulfonamidas.

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2.4 Estudio de la presencia de fármacos en aguas residuales urbanas de la provincia de Castellón

2.4.1 Introducción

Como se ha explicado en el Capítulo 1, los fármacos pueden llegar al medio ambiente a través de diversas vías de contaminación (Figura 1.2). Sin duda, la más importante de todas ellas es mediante su excreción y eliminación a través de las aguas residuales urbanas.

El comportamiento de los fármacos en las EDAR es complejo y desigual. Algunos experimentan degración química o biológica durante el proceso de depuración y/o debido a la acción de la luz solar (fotodegradación), otros se adsorben sobre los lodos de las plantas depuradoras, mientras que un elevado número de compuestos resisten a los tratamientos de depuración aplicados. Su comportamiento depende fundamentalmente de las propiedades físico-químicas de cada compuesto (estructura química, solubilidad acuosa, coeficiente de partición octanol/agua, etc.) y del tipo de tratamiento empleado (Jones, 2005).

Algunas sustancias pueden degradarse biológicamente durante su transporte a la EDAR, aunque lo más probable es que la degradación se produzca durante el tratamiento de depuración, en concreto cuando el compuesto se expone a concentraciones elevadas de microorganismos. Aquellas moléculas que poseen largas cadenas enlazadas tienen menor tendencia a la biodegradación que los compuestos con cadenas cortas. Los compuestos alifáticos saturados o los que poseen anillos aromáticos complicados y grupos sulfato o halógeno sufren una menor degradación y, por tanto, permanecen en el agua (Rogers, 1996).

Los compuestos en función de su polaridad pueden pasar a la fase acuosa o quedarse adsorbidos en las partículas sólidas (Figura 2.6) (Fatta, 2007). En general, los compuestos más hidrofílicos tienen tendencia a permanecer en la fase acuosa mientras que los más hidrofóbicos se acumulan en la fase sólida (lodos y fangos).

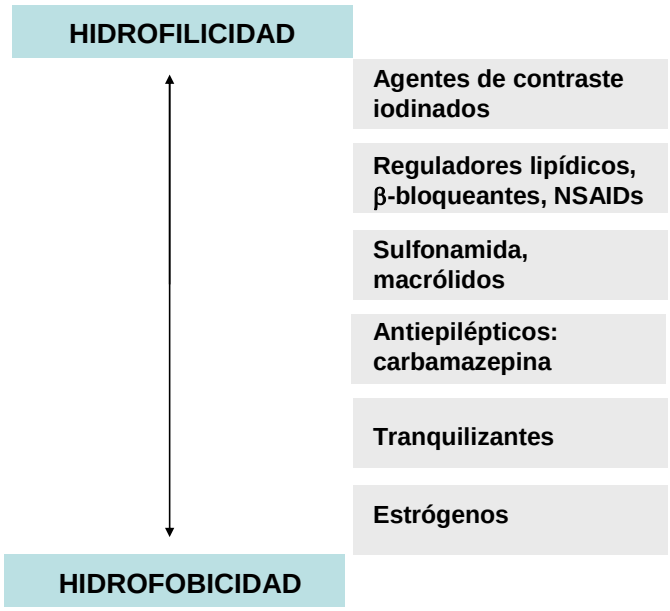

Figura 2.6 Nivel de hidrofilicidad e hidrofobicidad de los fármacos. Modificado de Fatta, 2007.

Es posible predecir la tendencia de adsorción de los compuestos a partir del coeficiente de partición octanol/agua (log K_{ow}). Los compuestos que se adsorben en los lodos poseen un log K_{ow} elevado (>4); por el contrario, aquellas sustancias con valores bajos (log K_{ow} < 2.5) presentan una mayor tendencia a permancer en el agua (Rogers, 1996).

Otro parámetro que puede ayudar a predecir el comportamiento de un compuesto es el log K_{oc} (coeficiente de carbón orgánico). Cuanto mayor es su valor, mayor es la probabilidad de que un compuesto se adsorba a la materia orgánica, por ejemplo, los sólidos supendidos, las grasas o los surfactantes presentes en las aguas residuales domésticas (Krogmann, 1999).

La afinidad de los compuestos a adsorberse en el lodo se representa mediante la constante de adsorción K_d. Así, cuanto mayor es el log K_d, mayor es la tendencia a transferirse de la fase acuosa al lodo (Le-Minh, 2010).

La mayoría de los fármacos son polares, relativamente hidrofílicos y tienen log K_{ow} bajo. Por ese motivo se encuentran principalmente en el agua. Sin embargo, aunque la tendencia a acumularse en los lodos de las depuradoras es baja, no debe ignorarse.

Antes de proseguir con esta discusión conviene discutir algunos aspectos de interés. El primero de ellos es el de "eliminación" de un compuesto, que hace referencia a que dicho compuesto deja de detectarse mediante un análisis específico en la fase en la que se encontraba (por ejemplo, en la fase acuosa). Esta eliminación ocurre como resultado de distintos procesos que pueden ser bióticos, como la biodegradación anteriormente explicada, o abióticos, tales como la hidrólisis, fotólisis, oxidación, reducción o adsorción (Kümmerer, 2009).

El término "eficacia de eliminación" de una EDAR para un compuesto dado se emplea en la literatura cuando se compara la concentración del compuesto en el influente y en el efluente (Miège, 2009), estableciendo el porcentaje de reducción de los niveles del compuesto en agua. Es importante señalar que una buena eficacia de eliminación no implica necesariamente que el compuesto se haya degradado. Para poder determinar el grado real de degradación se debería considerar también su concentración en el lodo o en las partículas suspendidas. Sin embargo, esta práctica es poco habitual posiblemente dada la complejidad que supone la toma de muestras de lodos y su posterior análisis (Miège, 2009; Jelic, 2011). En la actualidad hay pocos estudios publicados sobre la presencia de los fármacos en los lodos y en los sólidos suspendidos pero los datos disponibles indican que estos contaminantes también están presentes, en mayor o menor medida, en dichas matrices sólidas. Sin duda, se abre una línea interesante de investigación en este medio.

Otro de los factores de los que depende la eliminación de los fármacos es el tipo de tratamiento utilizado en la EDAR. La gran mayoría de los estudios publicados sobre la eliminación de fármacos se basan en la aplicación de tratamientos convencionales. Éstos se conocen como tratamientos primarios y secundarios y, generalmente, se dan uno seguido del otro. El tratamiento primario consiste en la sedimentación de los materiales suspendidos utilizando tratamientos físicos o físico-químicos. En algunos

casos, simplemente se dejan las aguas residuales en grandes tanques durante un cierto tiempo o, en el caso de los tratamientos primarios mejorados, a los tanques se les añaden sustancias químicas floculantes que aceleran la sedimentación.

El tratamiento secundario más habitual es un proceso biológico en el que el efluente proveniente del tratamiento primario se mezcla con agua cargada de lodos activos (microorganismos) para eliminar la materia orgánica biodegradable presente en el agua. Posteriormente, este líquido pasa a unos tanques en los que se produce la decantación de los lodos, dando como resultado un agua con menos contaminantes.

La eficacia de eliminación de los compuestos viene determinada por las condiciones de funcionamiento de la EDAR, teniendo especial influencia la temperatura, el tiempo de retención de los sólidos (*solid retention time*, SRT), el tiempo de retención hidráulica (*hydraulic retention time*, HRT) y la edad del lodo activo. Considerando la influencia de estas variables, que dependerán de cada EDAR, resulta difícil predecir el comportamiento de un compuesto durante el proceso de tratamiento. Además, la eficacia de eliminacion parace ser muy variable.

Dado que los tratamientos convencionales para la eliminación de contaminantes orgánicos tienen una capacidad muy variable y en ocasiones insuficiente, se necesita una mejora de los mismos utilizando tratamientos más avanzados. La filtración con arena, filtración a través de membranas de ósmosis inversa o de nanofiltración, tratamientos de adsorción utilizando carbón activo o resinas de intercambio iónico para eliminar contaminantes aniónicos y los procesos de oxidación química y fotoquímica (cloración, ozonización, radiación UV) son los tratamientos avanzados más utilizados hasta la fecha (Le-Minh, 2010). Estos tratamientos se aplican tras el tratamiento secundario y, aunque los estudios publicados a día de hoy no son muy numerosos, todos ellos coinciden en que mejoran la calidad del agua. Parece ser que los procesos de ozonización y cloración son los más efectivos cuando funcionan bajo las circunstancias óptimas (Le-Minh, 2010).

A pesar de las ventajas que aportan los tratamientos avanzados, su uso hoy en día está poco generalizado. El principal motivo es el elevado coste que supone su

implantación y los gastos operacionales, en cuanto al consumo de energía, que conllevan. Por lo general, las EDAR que disponen de tratamiento avanzado lo utilizan únicamente en ocasiones concretas, por ejemplo, cuando el agua tratada se reutiliza para el riego o en zonas declaradas sensibles (ej. humedales).

El artículo que se presenta a continuación es un estudio sobre la presencia de un número notable de fármacos, pertenecientes a las familias químicas más representativas, en las aguas residuales urbanas. Se seleccionaron tres depuradoras situadas en la provincia de Castellón. El muestreo se llevó a cabo en dos fases distintas, cubriendo en total las cuatro estaciones del año. La selección de los fármacos de la primera campaña (junio 2008 y enero 2009) se realizó sobre la base de su consumo con receta médica en España y su análisis se llevó a cabo mediante el método desarrollado en el *artículo científico 1*. En el segundo muestreo, realizado durante los meses de abril y octubre de 2009, se añadió a la lista un elevado número de antibióticos dado el interés que existe en torno a estos compuestos por sus posibles efectos peligrosos en el medio ambiente. Su determinación se efectuó mediante el método analítico presentado en el *artículo científico 2*. Como en el primer muestro, se tomaron muestras de influente y de efluente durante siete días consecutivos.

2.4.2 Artículo científico 3

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Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia

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ABSTRACT

A survey on the presence of pharmaceuticals in urban wastewater of a Spanish Mediterranean area (Castellon province) was carried out. The scope of the study included a wide variety of pharmaceuticals belonging to different therapeutical classes. For this purpose, 112 samples, including influent and effluent wastewater, from different conventional wastewater treatment plants were collected. Two monitoring programmes were carried out along several seasons. The first was in June 2008 and January 2009, and the second in April and October 2009. During the first monitoring, the occurrence of 20 analytes in 84 urban wastewater samples (influent and effluent) was studied. The selection of these pharmaceuticals was mainly based on consumption. From these, 17 compounds were detected in the samples, with analgesics and anti-inflammatories, cholesterol lowering statin drugs and lipid regulators being the most frequently detected groups. 4-Aminoantipyrine, bezafibrate, diclofenac, gemfibrozil, ketoprofen, naproxen and venlafaxine were the compounds most frequently found. In the highlight of these results, the number of analytes was increased up to around 50. A lot of antibiotic compounds were added to the target list as they were considered "priority pharmaceuticals" due to their more potential hazardous effects in the aquatic environment. Data obtained during the second monitoring programme (spring and autumn) corroborated the results from the first one (summer and winter). Analgesics and anti-inflammatories, lipid regulators together with quinolone and macrolide antibiotics were the most abundant pharmaceuticals. Similar median concentrations were found over the year and seasonal variation was not clearly observed. The removal efficiency of pharmaceuticals in the wastewater treatment plants was roughly evaluated. Our results indicated that elimination of most of the selected compounds occurred during the treatment process of influent wastewater, although it was incomplete.

1. Introduction

Pharmaceutical consumption is continuously increasing around the word. Only in Spain, about 729 millions of prescriptions were sold in 2004. Six years later, the consumption increased around 30% reaching 958 millions prescriptions (http://www.msps.es/profesionales/farmacia/datos/home.htm). This has lead to an increasing concern regarding possible ecological risks coming from pharmaceuticals released into the environment.

Pharmaceuticals are used extensively in human and veterinary medicine to prevent illness and also as growth promoters in livestock and fish farming as well as in agriculture. After administration, pharmaceuticals can be transformed in the human body into more polar and soluble forms as metabolites or as conjugates of glucuronic and sulphuric acid (Heberer, 2002; Nikolaou et al., 2007). Pharmaceuticals and their metabolites are readily excreted with urine and faeces and enter into urban wastewater treatment plants (WWTPs). Some of these compounds are eliminated by chemical or biological processes while others are degraded during sewage treatment processes or removed from the water phase by adsorption onto solid phase (e.g. sludge) (Jones et al., 2005). Data recently reported show that some pharmaceuticals are accumulated in sewage sludge. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent. In general, the elimination of most of the substances is incomplete and improvements of the wastewater treatment and subsequent treatments of the produced sludge are required to prevent the introduction of these micro-pollutants in the environment (Jelic et al., 2011). At present, urban wastewaters are considered the most important source of pharmaceutical compounds in the aquatic environment. WWTPs were designed to remove organic pollutants, mainly estimated as dissolved organic matter, solids and nutrients but not pharmaceutical compounds. Disposal of unused pharmaceuticals directly into domestic waste and application to livestock as veterinary drugs and feed additives can also

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Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia

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ABSTRACT

A survey on the presence of pharmaceuticals in urban wastewater of a Spanish Mediterranean area (Castellon province) was carried out. The scope of the study included a wide variety of pharmaceuticals belonging to different therapeutical classes. For this purpose, 112 samples, including influent and effluent wastewater, from different conventional wastewater treatment plants were collected. Two monitoring programmes were carried out along several seasons. The first was in June 2008 and January 2009, and the second in April and October 2009. During the first monitoring, the occurrence of 20 analytes in 84 urban wastewater samples (influent and effluent) was studied. The selection of these pharmaceuticals was mainly based on consumption. From these, 17 compounds were detected in the samples, with analgesics and anti-inflammatories, cholesterol lowering statin drugs and lipid regulators being the most frequently detected groups. 4-Aminoantipyrine, bezafibrate, diclofenac, gemfibrozil, ketoprofen, naproxen and venlafaxine were the compounds most frequently found. In the highlight of these results, the number of analytes was increased up to around 50. A lot of antibiotic compounds were added to the target list as they were considered “priority pharmaceuticals” due to their more potential hazardous effects in the aquatic environment. Data obtained during the second monitoring programme (spring and autumn) corroborated the results from the first one (summer and winter). Analgesics and anti-inflammatories, lipid regulators together with quinolone and macrolide antibiotics were the most abundant pharmaceuticals. Similar median concentrations were found over the year and seasonal variation was not clearly observed. The removal efficiency of pharmaceuticals in the wastewater treatment plants was roughly evaluated. Our

results indicated that elimination of most of the selected compounds occurred during the treatment process of influent wastewater, although it was incomplete.

Keywords

Pharmaceuticals; Liquid chromatography-tandem mass spectrometry; Effluent and influent wastewater; Waste water treatment plant; Occurrence; Removal efficiency.

1. Introduction

Pharmaceutical consumption is continuously increasing around the word. Only in Spain, about 729 millions of prescriptions were sold in 2004. Six years later, the consumption increased around 30% reaching 958 millions prescriptions (http://www.msps.es/profesionales/farmacia/datos/home.htm). This has lead to an increasing concern regarding possible ecological risks coming from pharmaceuticals released into the environment.

Pharmaceuticals are used extensively in human and veterinary medicine to prevent illness and also as growth promoters in livestock and fish farming as well as in agriculture. After administration, pharmaceuticals can be transformed in the human body into more polar and soluble forms as metabolites or as conjugates of glucuronic and sulphuric acid (Heberer, 2002; Nikolaou et al., 2007). Pharmaceuticals and their metabolites are readily excreted with urine and faeces and enter into urban wastewater treatment plants (WWTPs). Some of these compounds are eliminated by chemical or biological processes while others are degraded during sewage treatment processes or removed from the water phase by adsorption onto solid phase (e.g. sludge) (Jones et al., 2005). Data recently reported show that some pharmaceuticals are accumulated in sewage sludge. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent. In general, the elimination of most of the substances is incomplete and improvements of the wastewater treatment and subsequent treatments of the produced sludge are required to prevent the introduction of these micro-pollutants in the environment (Jelic et al., 2011). At present, urban wastewaters are considered the most important source of pharmaceutical compounds in the aquatic environment. WWTPs were designed to remove organic pollutants, mainly estimated as dissolved organic matter, solids and nutrients but not pharmaceutical compounds. Disposal of unused pharmaceuticals directly into

domestic waste and application to livestock as veterinary drugs and feed additives can also contribute to their introduction in the environment (Heberer, 2002; Nikolaou et al., 2007).

Removal efficiencies in WWTPs depend on several factors such as compound physico-chemical properties, the climate conditions (e.g. temperature and sunlight intensity), the type of treatment process employed, the operational conditions of the treatment process (temperature of operation, redox conditions, solids retention time and hydraulic retention time) as well as the age of the activated sludge used in the plant (Castiglioni et al., 2006; Suárez et al., 2008; Le-Minh et al., 2010). Therefore, removal efficiencies can vary significantly from plant to plant and within a plant at different time periods (Vieno et al., 2007).

WWTPs typically employ conventional sewage treatment consisting on primary sedimentation followed by secondary treatment and final sedimentation. Organic pollutants can be transformed from the aqueous phase by hydrolysis, biotransformation or sorption to primary and secondary sludges (Le-Minh et al., 2010). However, the removal efficiency is variable as it is highly affected by the compound affinity to remain in the aqueous phase of the treated effluent (hydrophilic pharmaceuticals) or to be adsorbed to sludge (hydrophobic chemicals). In contrast, tertiary treatment or advanced treatment processes such as membrane filtration, activated carbon or oxidative processes (chlorination, ozonation and ultraviolet irradiation) seem to be more efficient when they work under optimum conditions. Nevertheless, their use is not widespread due to their high cost in terms of energy consumption.

Little is known about possible human and ecological adverse effects derived from the presence of pharmaceuticals in the aquatic environment. Although the concentration levels detected after wastewater treatment processes seem not to cause toxic effects on human health and in the aquatic environment, there is a big concern on the long-term exposure of aquatic organisms to pharmaceuticals. Antibiotics are of special interest because they can promote bacterial resistance in the environment due to continuous exposure (Kümmerer, 2009a, 2009b; Zuccato et al., 2010). It is a problematic issue for flora and fauna as well as for humans, especially in those places where treated effluents are used to supplement drinking water supplies (Le-Minh et al., 2010). Consumption on antibiotics varies from country to country. Spain is one of the most consuming countries in terms of total amount. Broad spectrum antibiotics, which have the greatest impact on the development of resistance, are widely consumed according to the European Surveillance of Antimicrobial Consumption (ESAC) homepage (http://app.esac.ua.ac.be/public/index.php/ en_eu/antibiotic/ antibiotic-consumption).

The aim of this paper is to investigate the occurrence and behavior of pharmaceuticals in wastewater treatment plants placed in the Castellon province (Spanish Mediterranean area) in order to have a realistic knowledge of the presence of pharmaceuticals in this region. A total of 112 samples (untreated and treated urban wastewater samples) from three WWTPs were analyzed by liquid chromatography coupled to tandem MS, along two monitoring programmes over the four seasons: summer (June), winter (January), spring (April), and autumn (October). Up to 47 pharmaceuticals were determined including a notable number of antibiotics. The occurrence and removal of these pharmaceuticals in different WWTPs and the effect of the seasonal variation on the elimination of pharmaceuticals was assessed.

2. Experimental

2.1. Reagents and chemicals

Reference standards were purchased from Sigma–Aldrich (St Louis, MO, USA), LGC Promochem (London, UK), Toronto Research Chemicals (Ontario, Canada), Across Organics (Geel, Belgium), Bayer Hispania (Barcelona, Spain), Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid Spain) and Aventis Pharma (Madrid, Spain).

Isotopically labeled compounds used were omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, salicylic acid-d_3 and ibuprofen-d_3, from CDN Isotopes (Quebec, Canada); atorvastatin-d_5, paroxetine hydrochloride-d_4 and olanzapine-d_3, from Toronto Research Chemicals (Toronto, Canada); sarafloxacin-d_8 hydrochloride trihydrate, from Sigma–Aldrich; and sulfamethoxazole-$^{13}C_6$ and trimethoprim-$^{13}C_3$, from Isotope Cambridge Laboratories (Andover, MA, USA).

HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained from purification of demineralised water in a Milli-Q Gradient A10 (Millipore, Bedford, MA, USA). Formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac, reagent grade) and sodium hydroxide (NaOH, >99%) were supplied by Scharlab (Barcelona, Spain).

Standards were dissolved in MeOH, except macrolides, sulfonamides and lincosamides that were prepared in ACN. The addition of NaOH was necessary for the proper dissolution of acidic analytes like quinolones. A mix of all compounds was prepared in MeOH and

subsequently diluted with water to obtain working standard solutions. A mix of isotopically labeled internal standards (ILISs) was also prepared in MeOH and used as surrogate. All standard solutions and ILIS mix were stored in amber glass bottles at −20 °C in a freezer.

Cartridges used for SPE were Oasis HLB (60 mg) from Waters (Milford, MA, USA).

2.2. Instrumentation

Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC) analysis was carried out using an Acquity UPLC system (Waters, Milford, MS, USA), equipped with a binary solvent pumping. In the first monitoring, chromatographic separation of the 20 pharmaceuticals was achieved using an Acquity UPLC BEH column, 1.7 μm, 50 mm × 2.1 mm (i.d.) (Waters). Later, when the number of compounds increased up to 47, a longer column (Acquity UPLC HSS T3, 1.8 μm, 100 mm × 2.1 mm (i.d.)) was required for a satisfactory separation of all analytes but maintaining similar chromatographic runs. The LC system was interfaced to a TQD (triple quadrupole) mass spectrometer with an orthogonal electrospray ionization source Z-spray (Waters Corp.). MS/MS analysis was performed under selected reaction monitoring (SRM) mode, working in positive and negative ionization modes simultaneously. Chromatographic and mass spectrometry conditions can be found in detail in our previous papers (Gracia-Lor et al., 2010, 2011).

2.3. Analytical procedure

Water samples were extracted as described in Gracia-Lor et al. (2010, 2011). Briefly, the procedure was as follows: 100 mL water sample (100 mL effluent wastewater (EWW) or 20 mL influent wastewater (IWW) diluted with water to 100 mL) spiked with the ILIS mix working solution was passed through the Oasis HLB cartridge, previously conditioned. Analytes were eluted with 5 mL MeOH and the extract was evaporated and reconstructed with 1 mL MeOH–water (10:90, v/v). Finally, 20 μL of the final extract were injected in the UHPLC–MS/MS system. Quantification was made using calibration standards prepared in solvent, based on relative responses analyte/ILIS or on absolute analyte responses, depending on whether ILIS was used for correction or not. All methods applied were previously validated (Gracia-Lor et al., 2010, 2011).

2.4. Sampling

EWW and IWW samples were collected along 2008 and 2009. They were obtained from three WWTPs (Castellon de la Plana, Benicassim and Burriana) of the Castellon province (Spanish Mediterranean area). These WWTPs are designed to treat wastewaters (urban o mixed urban and industrial) operating with secondary treatment using conventional activated sludge. At present, the Castellon de la Plana WWTP has a tertiary treatment operating with sand filtration and ultraviolet irradiation, but it was not operating when the monitoring was carried out. Castellon de la Plana WWTP has a population equivalent of 265,000 inhabitants, while Benicassim and Burriana WWTPs serve to a population around 18,000 and 35,000 inhabitants. For each plant, 24-h composite untreated (influent) and treated wastewater samples (effluent) were obtained. Samples were frozen and stored at −18 °C until analysis.

Sampling was carried out in two campaigns. In the first monitoring, samples were collected along one complete week in June 2008 and in January 2009 and the occurrence of 20 pharmaceuticals was investigated (Gracia-Lor et al., 2010). In the second monitoring, in the light of the results obtained, the number of investigated compounds was increased up to 47 in order to have a wider knowledge of the presence of pharmaceuticals in wastewaters. Most of pharmaceuticals added in the second monitoring corresponded to antibiotics. In this case, only EWW and IWW samples from the Castellon de la Plana WWTP (the main town of the Castellon province) were analyzed as no significant differences between the three studied WWTPs were observed and this treatment plant serves a larger population. 24-h Composite samples (IWW and EWW) were collected during one complete week in April 2009 and October 2009.

3. Results and discussion

3.1. First monitoring

First of all, a group of 20 pharmaceuticals were selected including the most consumed active principles with medical prescription in Spain (Ministry of Health, 2008, 2009). Several compounds with low official sales volumes (in terms of medical prescription) but frequently detected in urban wastewater as reported by other authors (Ternes, 2001; Gros et al., 2006; Hernando et al., 2007; Pedrouzo et al., 2007) were also included (e.g. diclofenac, naproxen or bezafibrate). In addition, two metabolites were considered: salicylic acid, which is the main metabolite of acetylsalicylic acid, and 4-aminoantipyrine, which is a metabolite of dipyrone.

These metabolites were selected because they had been frequently determined in the aquatic environment (surface water and wastewater) according to scientific literature (Ternes et al., 2001; Heberer, 2002; Metcalfe et al., 2003; Wiegel et al., 2004). Thus, 20 pharmaceuticals for human use were selected (Table 1). Target analytes represented a broad range of chemicals classes including analgesic and anti-inflammatory, cholesterol lowering statin drugs, lipid regulators, antidepressants, anti-ulcer agents, psychiatric drugs, ansiolitics and cardiovasculars.

In total, 84 wastewater samples were analyzed in this monitoring, and collected from three WWTPs of the Castellon province. Sample collection was performed in summer 2008 (June) and winter 2009 (January). Table 1 shows the percentage of positive findings of the selected compounds, as well as median concentrations in IWW and EWW analyzed during this period.

13 out of 20 compounds were detected in IWW. All 13 pharmaceuticals were identified in more than 95% of the samples, with the exception of salicylic acid and pravastatin, the latest only being present in 26% of IWW samples. Analgesics/anti-inflammatories and lipid regulators were the most commonly detected groups. Moreover, the highest values in this type of samples corresponded to salicylic acid, acetaminophen and ibuprofen (these three compounds belong to the anti-inflammatory therapeutic group) with maximum levels of 277, 201 and 40 μg L^{-1}, and median concentration of 35.1, 44.8 and 12.4 μg L^{-1}, respectively. Quantification of the samples with high analyte levels (typically above 100 μg L^{-1}) required an additional analysis with previous dilution of the sample before the SPE step.

When comparing the percentage of positive findings in IWW collected in summer and in winter, no relevant differences were found. However, when comparing the maximum levels found, higher concentrations were observed for some compounds in the winter samples. For example, in the case of acetaminophen, salicylic acid and ibuprofen, maximum concentrations increased from 84 to 201 μg L^{-1}, from 47 to 277 μg L^{-1}, and from 20 to 40 μg L^{-1}, respectively. For the rest of compounds, no relevant variations in concentrations were observed.

Table 1
Summary of the results obtained in the first monitoring programme of 20 pharmaceuticals (June 2008 and January 2009).

	Therapeutic group	Influent wastewater (n = 42)				Effluent wastewater (n = 42)			
		% Positive findings	Median concentration (µg/L)	Minimum-Maximum levels (µg/L)	LOQ (µg L-1)[a]	% Positive findings	Median concentration (µg/L)	Minimum-Maximum levels (µg/L)	LOQ (µg L-1)[a]
Acetaminophen	Analgesics and anti-inflamatories	100	44.8	1.13-201	0.11	0	n.d.	n.d.	0.09
4-Aminoantipyrine		100	2.26	0.53-6.45	0.03	100	0.69	0.42-1.68	0.04
Diclofenac		100	0.56	0.26-1.49	0.14	100	0.33	0.06-0.74	0.05
Ibuprofen		98	12.4	2.28-39.8	0.64	33	<LOQ	<LOQ	0.25
Ketoprofen		100	0.48	<LOQ-1.17	0.11	100	0.30	0.15-0.62	0.07
Naproxen		100	1.55	0.27-3.58	0.05	100	0.17	<LOQ-0.72	0.03
Salicylic acid		76	35.1	3.10-277	0.97	26	<LOQ	<LOQ-236	0.43
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	100	0.11	<LOQ-0.45	0.03	76	0.02	0.01-0.16	0.007
Pravastatin		26	0.20	0.14-0.24	0.12	30	0.10	0.07-0.17	0.02
Bezafibrate		100	0.16	0.02-0.46	0.02	100	0.07	0.02-0.39	0.01
Gemfibrozil		100	1.11	0.16-2.12	0.05	100	0.54	0.15-1.24	0.02
Paroxetine	Antidepressants	0	n.d.	n.d.	0.20	0	n.d.	n.d.	0.04
Venlafaxine		100	0.17	0.04-0.52	0.01	100	0.14	0.06-0.30	0.004
Omeprazole	Anti-ulcer agents	0	n.d.	n.d.	0.03	0	n.d.	n.d.	0.01
Pantoprazole		0	n.d.	n.d.	0.07	65	0.13	0.05-0.18	0.03
Olanzapine	Psychiatric drugs	0	n.d.	n.d.	0.01	0	n.d.	n.d.	0.01
Risperidone		0	n.d.	n.d.	0.009	0	n.d.	n.d.	0.006
Alprazolam	Ansiolitics	0	n.d.	n.d.	0.03	38	<LOQ	<LOQ	0.01
Lorazepam		0	n.d.	n.d.	0.05	55	0.04	0.03-0.06	0.03
Enalapril	Cardiovascular	96	0.14	0.02-0.29	0.02	0	n.d.	n.d.	0.007

n.d. (not detected)

[a] Data on LOQ taken from (Gracia-Lor et al., 2010).

Regarding EWW, up to 14 target compounds were detected. Analgesic and anti-inflamatories were frequently found (the exception was acetaminophen, which was never detected in the EWW samples in contrast to IWW where it was present in the 100% of samples). Cholesterol lowering statin drugs and lipid regulators were also found in a high number of samples but, with the exception of gemfibrozil, their median concentrations were below 0.10 μg L^{-1}. Other compounds frequently detected were venlafaxine, pantoprazole and lorazepam.

The removal of pharmaceuticals during wastewater treatment was estimated from concentration data in IWW and EWW. Considering that pharmaceuticals have rather different physico-chemical characteristics, their removal during treatment is expected to be diverse. In the literature, the removal efficiency is generally computed as the percentage of reduction between the dissolved aqueous phase concentration of the contaminant in the influent and the dissolved aqueous phase concentration of the contaminant in the effluent. Except for a few studies, pharmaceutical concentrations in sludge or suspended solid are generally not considered nor measured, probably because of the difficulty to sample and to analyze such complex matrices (Miège et al., 2009). However, the screening of sewage sludge showed that these micro-pollutants are very present in this medium. This indicates that even good removal rates obtained in aqueous phase (i.e. comparison of influent and effluent wastewater concentrations) do not imply degradation to the same extent (Jelic et al., 2011). When comparing pharmaceutical concentrations in IWW and EWW, like in this work, lower levels in EWW would be interpreted as a removal of the compound in the WWTP. This fact might be due to different factors like chemical and physical transformations, biodegradation and sorption to the solid matter. Thus, the conversion of a given pharmaceutical to compounds other than the analyzed one would lead to lower pharmaceutical levels in EWW concluding that an “apparent” removal takes place.

In this work, acetaminophen, enalapril and ibuprofen were completely removed during the treatment processes (present in 100% and 96% of IWW samples, and never detected in the EWW samples), while the antidepressant venlafaxine, lipid regulator compounds, as well as analgesic and anti-inflammatory pharmaceuticals (with the exception of acetaminophen and ibuprofen) were detected in all EWW samples, although at concentrations lower than in IWW. On the other hand, some pharmaceuticals were not detected in IWW but they were present in EWW. This behavior was observed for pantoprazole and for the ansiolitic compounds alprazolam and lorazepam. This is in agreement with previous studies where some compounds

were reported to be more abundant in effluents than in influents (Lacey et al., 2008; Gros et al., 2010; Jelic et al., 2011). In the case of the ansiolitic compounds, they were detected at very low concentrations in EWW (around or below the LOQ level). Maybe they were also present in the IWW samples but could not be detected due to the lower sensitivity of the method in this type of waters. The higher complexity of the influents leads to strong matrix effects (commonly ionization suppression), which can hamper the detection of some analytes at very low levels. The absence of ansiolitic compounds in the IWW might be also due to the enzymatic cleavage of the compound glucuronides and other conjugated metabolites and the subsequent release of the parent compound during the treatment process (Vieno et al., 2007; Lacey et al., 2008; Gros et al., 2010).

Predicting the removal efficiencies of compounds during treatment processes is quite difficult because they are significantly affected by the specific operating conditions of each WWTP. However, some information can be obtained from the data reported by others on the behavior of pharmaceuticals during the treatment processes. For instance, analgesics and anti-inflammatory pharmaceuticals have been detected in the aquatic environment in a broad number of studies. Within this group, our data showed that acetaminophen was removed by the three WWTPs. For salicylic acid, an efficient removal was also obtained in contrast to diclofenac, ketoprofen and naproxen that seemed to persist to the water treatment, although their levels in EWW were lower than in IWW. This behavior is consistent with scientific literature (Heberer, 2002; Gros et al., 2010).

In the case of lipid regulators and cholesterol lowering statin drugs, they showed a variety of removal rates between 30% and 100% which is in fairly good agreement with previous studies (Jelic et al., 2011). In our case, the highest levels and frequency of detection were found for lipid regulators, especially for gemfibrozil.

Comparing the three studied WWTPs, no significant differences in terms of removal efficiencies were observed for the analyzed compounds. This is because they work at similar operational conditions.

3.2. Second monitoring

A notable number of compounds (around 30 antibiotics and a cholesterol lowering statin drug) were added to the target list of our previous method in order to have a more realistic

knowledge of the presence of pharmaceuticals in the environment. Many antibiotics were included due to the special concern on their potential negative effects on the aquatic environment, whereas simvastatin, a cholesterol statin drug, was added to the list due to its increased consumption with medical prescription.

As differences among the three WWTPs were hardly observed in the first survey, in the second monitoring only the Castellon de la Plana WWTP was monitored, in two different seasons: spring (April 2009) and autumn (October 2009). This treatment plant was selected because it serves the largest population of the Castellon province (Table 2). Moreover, data obtained in the first monitoring revealed that the samples from this treatment plant typically presented the highest pharmaceutical levels. In this second monitoring, 28 wastewater samples (14 IWW and 14 EWW) were collected and analyzed (Table 3), corresponding to one whole week of April 2009 and one whole week of October 2009.

Table 2
Characteristics of the Castellon de la Plana treatment plant.

WWTP	Population (he)	Type of treatment	Type of wastewater treated	Designed treatment capacity (m^3d^{-1})	Average flow (m3/day)	Minimum flow estimated (L/s)	Maximum flow estimated (L/s)	Sampling
Castellon de la Plana	265,000	Secondary[a]	Urban and industrial	42,000	36,000	139.06	752.31	Time-proportional composite (every 60 minutes)

[a] Secondary treatment was applied at the time of the monitoring was performed. At present, a tertiary treatment is applied.

In IWW, for those 20 pharmaceuticals also analyzed in the first monitoring, no relevant differences were observed except for diclofenac, which showed a lower frequency of detection. Similarly to the previous study, the highest concentrations in IWW were found for acetaminophen (134 μg L^{-1}), salicylic acid (64 μg L^{-1}) and ibuprofen (19 μg L^{-1}). As pointed out before, these compounds are frequently prescribed but they can also be acquired without medical prescription, the so-called “over the – counter” (OTC) drugs.

In the case of antibiotics, it is difficult to establish a general trend for each group. As shown in Table 3, 9 out of 26 selected antibiotics were detected in the influent samples. Among them, seven compounds (ciprofloxacin, clarithromycin, norfloxacin, ofloxacin, pipemidic acid, sulfamethoxazole and trimethoprim) were detected in all the samples. On the contrary, lincomycin, which can be used in both human and veterinary medicine, and sulfathiazole were detected in around 20% of IWW. Except for ciprofloxacin, antibiotic median concentrations in IWW did not exceed 1 μg L^{-1}.

Regarding EWW, our data suggest that elimination of most of the compounds analyzed is incomplete. Again, we may distinguish different behaviors. First of all, there is a group of compounds that were fully eliminated in the treatment plant (i.e. acetaminophen, enalapril, ibuprofen, salicylic acid). These results are in agreement with those reported by other authors (Gros et al., 2010) and supported the behavior observed in the first monitoring, with slight differences observed for salicylic acid. On the other hand, there are several compounds partly removed by the treatment processes. For these compounds, concentrations after treatment were normally lower than in IWW, but they were still present in the EWW analyzed. This is the case of most lipid regulators and anti-inflammatory drugs. In some particular cases, e.g. gemfibrozil, concentrations were slightly higher in the effluent. Another group of pharmaceuticals included those compounds that showed poor or non elimination in the treatment plant, as some macrolide antibiotics, ansiolitics and the anti-ulcer agent pantoprazole, which presented even higher percentages of positive findings in EWW than in IWW. This fact has been previously reported in other studies (Göbel et al., 2007; Gros et al., 2010). As pointed out before, this phenomenon might be explained by the higher LOQs in IWW compared to EWW, or by the release of the parent compound from glucuronides or other conjugated metabolites during the treatment process. Finally, several target analytes were never found either in IWW or EWW. It was not expected for those compounds such as simvastatin, omeprazole or paroxetine that belong to the list of the most consumed pharmaceuticals in Spain with medical prescription. Their absence might be explained because their excretion was mainly as metabolites or due to the parent compound transformation/degradation in the sewer system. Thus, searching for metabolites and/or transformation products of these compounds seems necessary to evaluate their impact into the aquatic ecosystem.

Concerning sulfonamide antibiotics, only sulfamethoxazole was detected in EWW. In fact, it was present in 100% EWW analyzed although at very low levels, below 0.06 μg L^{-1}. Some contradiction exists about its removal (Le-Minh et al., 2010) as some studies have observed an effective removal (Choi et al., 2008) while others not (Brown et al., 2006). This fact might be explained by differences in operational conditions of each WWTP.

Table 3
Summary of the results obtained in the second monitoring programme of 47 pharmaceuticals (April 2009 and October 2009).

	Therapeutic group	Influent wastewater (n = 14)				Effluent wastewater (n = 14)			
		% Positive findings	Median concentration (μg L^{-1})	Minimum-Maximum levels (μg L^{-1})	LOQ (μg L^{-1})[a]	% Positive findings	Median concentration (μg L^{-1})	Minimum-Maximum levels (μg L^{-1})	LOQ (μg L^{-1})[a]
Acetaminophen	Analgesics and anti-inflamatories	100	55.1	18.2-134	0.19	0	n.d.	n.d.	0.09
4-Aminoantipyrine		100	2.30	0.90-3.20	0.03	100	0.74	0.56-0.95	0.02
Diclofenac		36	0.53	<LOQ-0.74	0.14	100	0.34	0.21-0.62	0.05
Ibuprofen		100	14.6	6.1-19.1	0.73	0	n.d.	n.d.	0.15
Ketoprofen		100	0.37	0.25-0.41	0.14	100	0.33	0.12-0.42	0.05
Naproxen		100	1.32	0.87-2.24	0.08	100	0.13	0.09-0.28	0.02
Salicylic acid		100	38.1	10.9-63.7	0.24	0	n.d.	n.d.	0.08
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	100	0.22	0.11-0.33	0.01	93	0.02	0.01-0.04	0.004
Pravastatin		50	<LOQ	<LOQ-0.25	0.20	0	n.d.	n.d.	0.03
Bezafibrate		100	0.08	<LOQ-0.10	0.03	100	0.06	0.04-0.08	0.01
Gemfibrozil		100	0.21	0.10-0.54	0.07	100	0.49	0.34-0.91	0.004
Simvastatin		0	n.d.	n.d.	0.13	0	n.d.	n.d.	0.02
Paroxetine	Antidepressants	0	n.d.	n.d.	0.27	0	n.d.	n.d.	0.17
Venlafaxine		100	0.87	0.78-0.98	0.05	100	0.29	0.20-0.55	0.007
Omeprazole	Anti-ulcer agents	0	n.d.	n.d.	0.06	0	n.d.	n.d.	0.02
Pantoprazole		0	n.d.	n.d.	0.06	100	0.01	0.01-0.02	0.004
Olanzapine	Psychiatric drugs	0	n.d.	n.d.	0.17	0	n.d.	n.d.	0.05
Risperidone		0	n.d.	n.d.	0.03	0	n.d.	n.d.	0.003
Alprazolam	Ansiolitics	0	n.d.	n.d.	0.03	100	0.01	0.01-0.01	0.004
Lorazepam		0	n.d.	n.d.	0.15	100	0.14	0.10-0.16	0.05
Enalapril	Cardiovascular	100	0.15	0.09-0.20	0.02	0	n.d.	n.d.	0.006
Erithromycin	Macrolide antibiotics	0	n.d.	n.d.	0.02	100	0.08	0.05-0.12	0.008
Clarithromycin		100	0.23	0.13-0.62	0.01	100	0.02	0.01-0.06	0.003
Tylosin		0	n.d.	n.d.	0.01	0	n.d.	n.d.	0.002
Roxithromycin		0	n.d.	n.d.	0.03	50	<LOQ	<LOQ	0.02
Moxifloxacin	Quinolone antibiotics	0	n.d.	n.d.	0.33	100	0.16	0.12-0.18	0.11
Norfloxacin		100	0.40	0.29-1.07	0.16	100	0.13	0.09-0.15	0.03
Pefloxacin		0	n.d.	n.d.	0.08	0	n.d.	n.d.	0.05
Ofloxacin		100	0.76	0.29-0.96	0.01	100	0.44	0.33-0.50	0.01
Marbofloxacin		0	n.d.	n.d.	0.12	0	n.d.	n.d.	0.11
Ciprofloxacin		100	2.45	1.21-3.85	0.32	100	0.70	0.52-1.08	0.05
Enrofloxacin		0	n.d.	n.d.	0.04	0	n.d.	n.d.	0.02
Sarafloxacin		0	n.d.	n.d.	0.04	0	n.d.	n.d.	0.03
Flumequine		0	n.d.	n.d.	0.06	0	n.d.	n.d.	0.01
Oxolinic acid		0	n.d.	n.d.	0.02	0	n.d.	n.d.	0.01
Nalidixic acid		0	n.d.	n.d.	0.02	0	n.d.	n.d.	0.006
Pipemidic acid		100	0.28	<LOQ-0.54	0.21	100	0.10	<LOQ-0.12	0.09
Sulfamethoxazole	Sulfonamide antibiotics	100	0.45	0.22-0.64	0.02	100	0.05	0.04-0.06	0.01
Sulfamethazine		0	n.d.	n.d.	0.003	0	n.d.	n.d.	0.001
Sulfadiazine		0	n.d.	n.d.	0.08	0	n.d.	n.d.	0.05
Sulfathiazole		29	0.06	0.06-0.07	0.04	0	n.d.	n.d.	0.01
Lincomycin	Lincosamide antibiotics	14	0.49	0.10-0.88	0.01	79	0.01	0.01-0.16	0.002
Clindamycin		0	n.d.	n.d.	0.04	100	0.02	0.01-0.02	0.006
Furaltadone	Other antibiotics	0	n.d.	n.d.	0.01	0	n.d.	n.d.	0.005
Furazolidone		0	n.d.	n.d.	0.02	0	n.d.	n.d.	0.02
Trimethoprim		100	0.10	0.06-0.16	0.04	100	0.09	0.06-0.10	0.01
Chloramphenicol		0	n.d.	n.d.	0.03	0	n.d.	n.d.	0.02

n.d. (not detected)
[a] Data on LOQ taken from (Gracia-Lor et al., 2011).

The presence of trimethoprim is usually related to the detection of sulfamethoxazole since these pharmaceuticals are often administered together. In agreement with other studies (Ternes, 2001; Gros et al., 2010; Jelic et al., 2011), the removal of trimethoprim during the wastewater treatment was incomplete.

In the case of macrolide antibiotics, all compounds belonging to this therapeutic group were detected in EWW, except for tylosin which was never found. Among them, the percentage of positive findings and concentrations may differ due to their different consumption pattern. The incomplete removal of macrolide antibiotics by WWTP is in agreement to previous works (Clara et al., 2005; Göbel et al., 2007). In our study, erithromycin and roxithromycin were present in the effluent samples, but absent in the corresponding influent. Some authors suggest that this might be due to the release of these compounds from faeces during the biological treatment (Göbel et al., 2007).

Regarding quinolone antibiotics, they have been frequently detected in wastewaters from several countries, especially norfloxacin and ciprofloxacin. In our case, 5 out of 12 compounds that belong to this group were found in EWW (see Table 3).

The results obtained in this monitoring work support the interest for including antibiotics when monitoring pharmaceuticals in wastewater, as they have been found rather frequently in the samples.

In all samplings carried out, samples were collected during one complete week. In general, concentration of pharmaceuticals did not significantly change along the week. This indicates that the consumption of the studied compounds is quite constant over the week in contrast to illicit drugs, which consumption clearly increases during the weekends and in special events (Bijlsma et al., 2009).

In this survey, a more complete seasonal variation analysis could be made for the Castellon de la Plana WWTP, which was the only one sampled in all monitoring programmes (four seasons, from summer 2008 to autumn 2009). Regarding the 20 most consumed pharmaceuticals, initially selected, they did not show big variations in median concentrations over the year (Fig. 1a and b). This is in accordance to their use, which is rather constant along the year. For a few compounds higher concentrations were found in winter for IWW (acetaminophen, salicylic acid, naproxen or diclofenac). These compounds are analgesic and

anti-inflammatory pharmaceuticals, which are consumed along the whole year, but especially in winter.

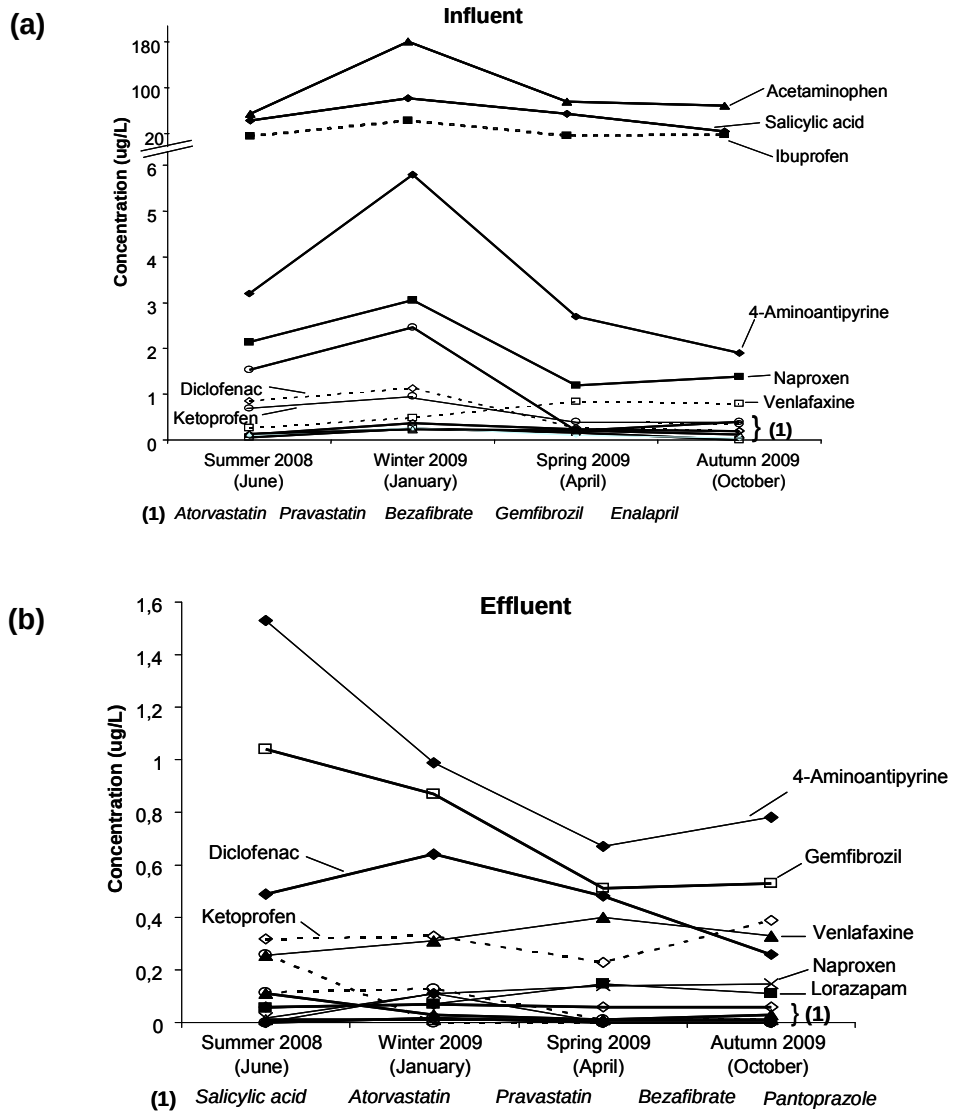

Figure 1. Median pharmaceutical concentration in the influent (a) and in the effluent (b) of the Castellon de la Plana WWTP monitored along four seasons.

Regarding antibiotics, a comparison between spring and autumn concentrations was made, as they were only determined in these two seasons (Fig. 2a and b). We did not observe relevant differences, as the same compounds were detected in both seasons at similar median

concentrations. However, it is noteworthy that antibiotic concentrations were notable lower than for the rest of pharmaceuticals, probably because they are less consumed. The only exception was ciprofloxacin.

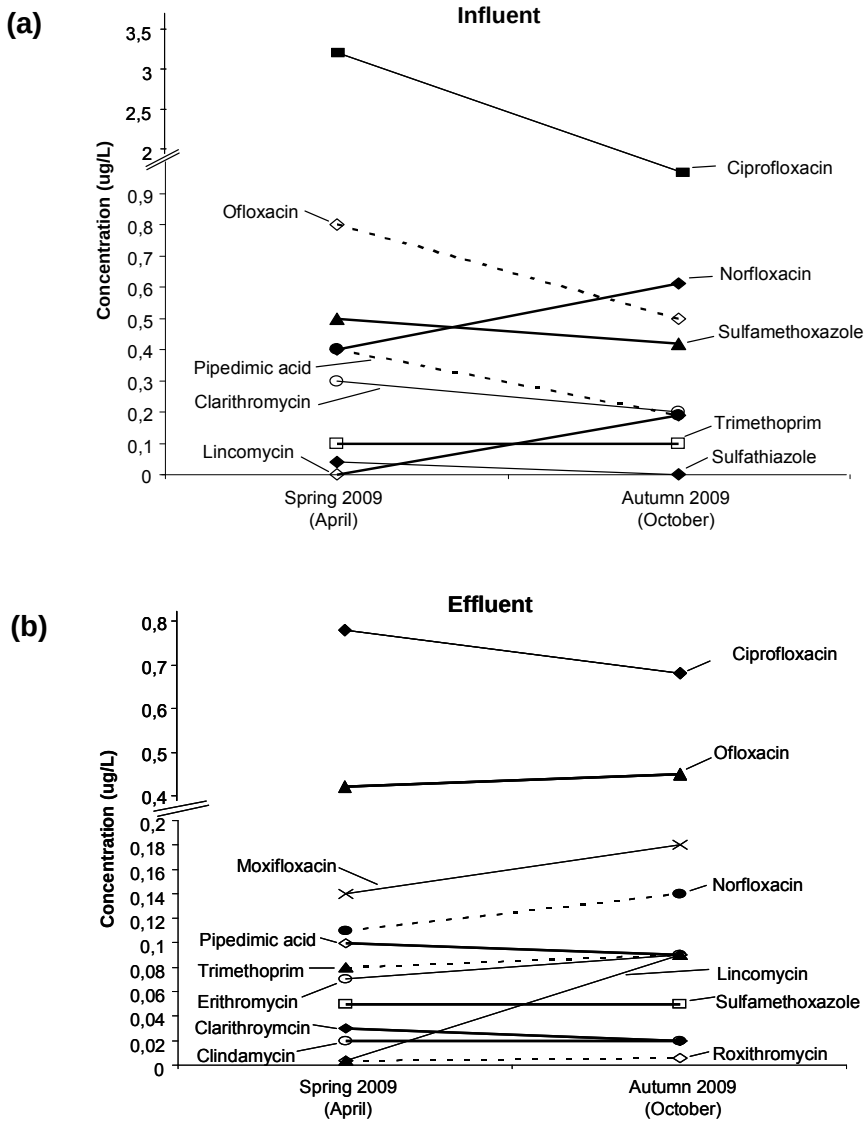

Figure 2. Median antibiotics concentration in the influent (a) and in the effluent (b) of the Castellon de la Plana treatment plant monitored in spring and autumn 2009.

The removal efficiency (RE) of the Castellon de la Plana WWTP is illustrated in Fig. 3. Those pharmaceuticals that were not detected in influent and in effluent wastewater samples

(e.g. simvastatin, paroxetine, pefloxacin, etc.) have been omitted in this figure. RE values were calculated as the ratio between the median concentration levels of each pharmaceutical in influents and effluents. Data from samples collected along a whole week in April 2009 have been used in this figure. This WWTP seemed to have good removal efficiency for most analgesics/anti-inflammatories like acetaminophen, ibuprofen, naproxen or salicylic acid (RE around 100%). As regards the four cholesterol lowering statin drugs/lipid regulators detected in wastewater, two of them seemed to be efficiently removed (atorvastatin, pravastatin), while partial removal was suggested for bezafibrate (RE around 40%) and no removal was observed for gemfibrozil. The cardiovascular enalapril was also efficiently removed.

In relation to antibiotics, 6 out of 13 compounds detected in wastewater were rather efficiently removed in the WWTP, whereas ofloxacin and trimethoprim showed RE between 20% and 40%. However, negative RE were observed for 5 antibiotics because these pharmaceuticals were not detected in IWW samples but were present in the corresponding EWW samples. In this case, it was not possible to calculate the RE actually, and a reference value (−100%) was given in order to show their behavior in the figure. The same situation was observed for three more compounds (pantoprazole, alprazolam and lorazepam) that were not found in IWW although they were detected in EWW (all compounds marked as (*) in Fig. 3). As previously stated, this situation might be due to the non-detection in IWW as a consequence of the higher complexity of this matrix, with typically higher matrix suppression, and the higher LOQs resulting in IWW. It must be taken into account that concentration levels found in EWW were normally low for all those compounds. Thus, they might be present at low levels in the IWW as well, and might not have been detected. Therefore, this assigned arbitrary value of -100% for al these 8 compounds might be questioned.

In the case of gemfibrozil and diclofenac, negative RE values were due to a slight increase of their concentration during the treatment process, i.e., they were detected at higher concentration levels in the effluent.

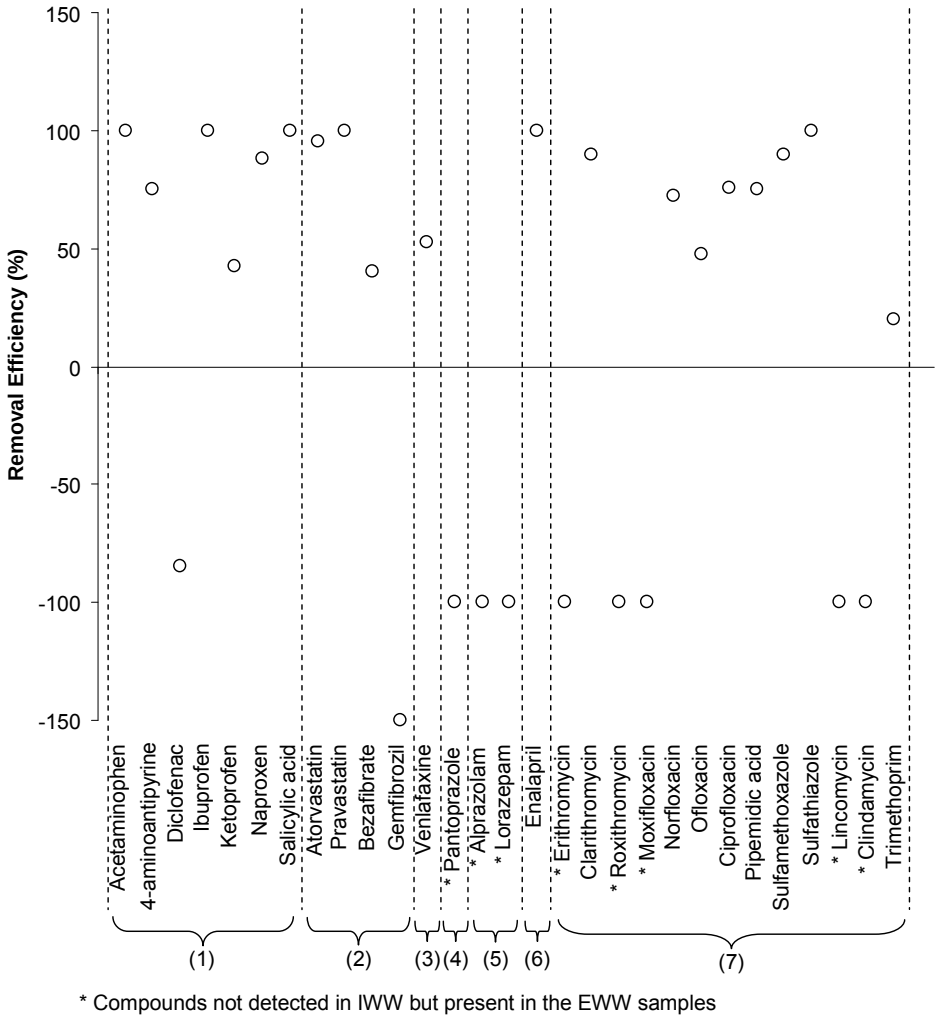

Figure 3. Removal efficiency of the Castellon de la Plana WWTP. (Data from April 2009). (1) Analgesics and anti-inflammatories, (2) cholesterol lowering statin drugs and lipid regulators, (3) antidepressants, (4) anti-ulcer agents, (5) ansiolitics, (6) cardiovasculars, and (7) antibiotics.

4. Conclusions

In this study, a monitoring of around 50 pharmaceuticals has been made in IWW and EWW from three different WWTPs. Up to 17 compounds were detected in both IWW and EWW indicating that conventional treatment processes do not completely remove these micro-

pollutants. Among them, analgesics and anti-inflamatories, lipid regulators as well as quinolone and macrolide antibiotics were the major groups found.

Selected pharmaceuticals could be divided into four groups according to their behavior in WWTPs: a few compounds were completely removed during the treatment processes (e.g. acetaminophen, enalapril, ibuprofen); another group of analytes were not fully removed, although their concentrations after treatment were significantly lower than in influent (e.g. lipid regulators). A third group of compounds were not detected in IWW but were present in the EWW samples (e.g. ansiolitics and macrolide antibiotics). Finally, some pharmaceuticals were never detected in either IWW or EWW (e.g. simvastatin, paroxetine, sulfamethazine).

Searching for metabolites may offer valuable information (Tarcomnicu et al., 2011), especially for those analytes never found in wastewater despite they were frequently used. Future research will be directed towards the investigation of metabolites by using quadrupole time of flight (QTOF) mass spectrometry. In those particular cases where pharmaceuticals were not detected in IWW but detected in the corresponding EWW samples, QTOF would also be an ideal approach to identify glucuronide and conjugated metabolites, if present in IWW. Thus, the occurrence of metabolites and conjugated compounds could be studied by this technique.

Seasonal variation in terms of median concentration values was not clearly observed in IWW and EWW. However, when comparing the maximum levels reached, higher concentrations were found in winter (January 2009), especially for analgesic and anti-inflamatory pharmaceuticals, possible due to a higher consumption during this period of the year to treat, for example, seasonal flu.

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2.4.3 Discusión de los resultados (artículo científico 3)

Primer muestreo

En el primer muestreo se estudió la presencia de 20 fármacos en un total de 84 muestras procedentes de tres EDAR situadas en la provincia de Castellón (Burriana, Benicassim y Castellón de la Plana). Las muestras se recogieron durante una semana completa en junio de 2008 y otra en enero de 2009.

Muestras de influente

Se analizaron 42 muestras y se detectaron 13 de los 20 fármacos estudiados. Once de ellos se identificaron en más del 95% de las muestras. Los compuestos pertenecientes al grupo de los analgésicos y antiinflamatorios y los reguladores lipídicos fueron los más detectados. El rango de concentraciones de los analitos fue muy amplio, especialmente en el caso de acetaminofeno y del ácido salicílico, con niveles de concentración entre 1.13–201 µg/L y 3.10-277 µg/L respectivamente. La concentración mediana de los analgésicos/antiinflamatorios fue superior a la del resto de compuestos detectados.

No se observaron diferencias significativas en la frecuencia de detección de los compuestos entre verano (junio 2008) e invierno (enero 2009). En cambio, como se puede ver en las siguientes gráficas (Figura 2.7), sí hubo diferencias en cuanto a los niveles de concentración que, en general, fueron superiores en las muestras recogidas en el mes de enero. Las diferencias más significativas se encontraron para los analgésicos/antiinflamatorios. Estos datos se comentarán más adelante.

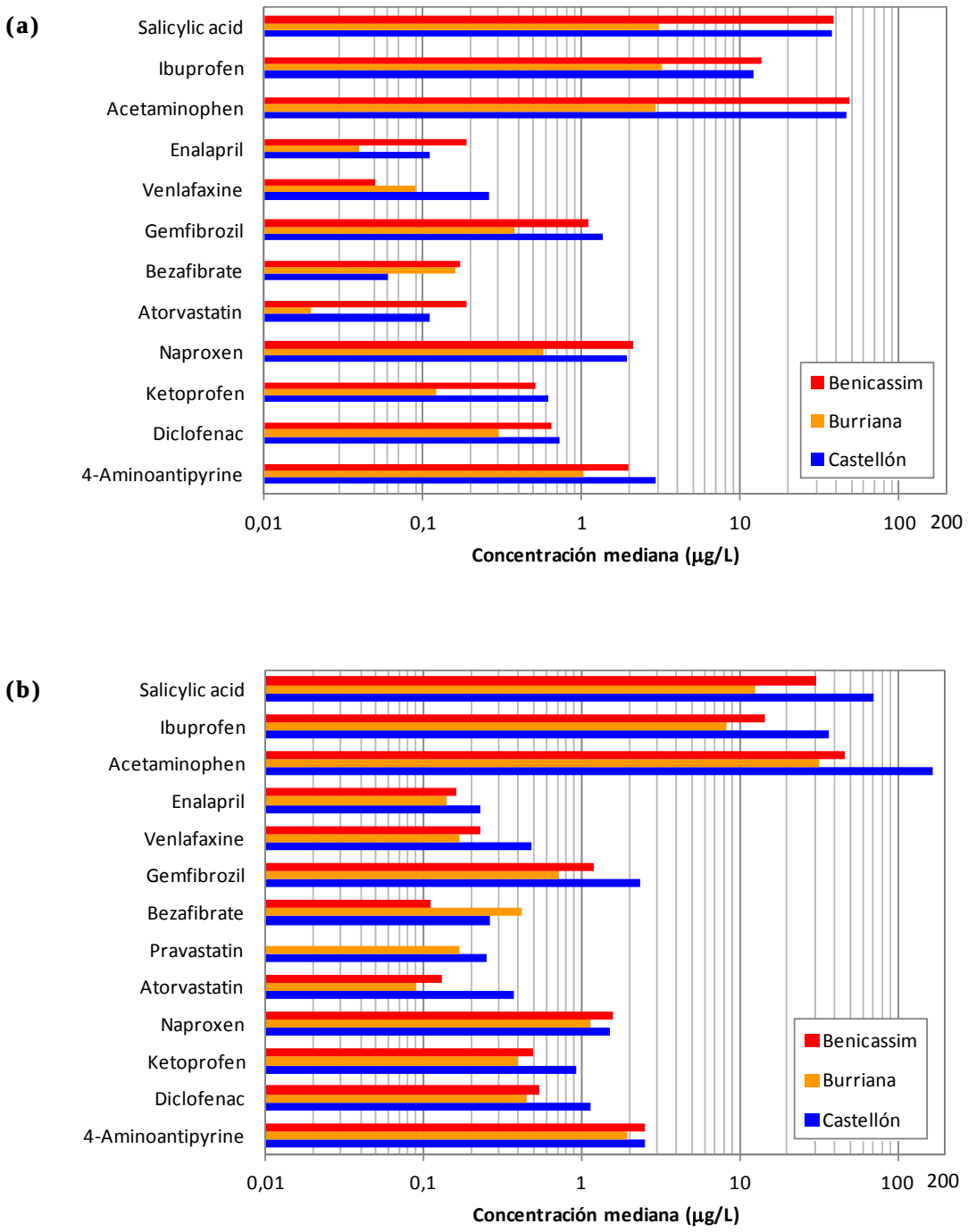

Figura 2.7 Concentración mediana (µg/L) de los fármacos encontrados en el influente de tres EDAR. (a) Junio 2008. (b) Enero 2009.

Muestras de efluente

Se detectaron 14 compuestos de los 20 seleccionados en las 42 muestras analizadas. Al igual que en las muestras de influente, los analgésicos/antiinflamatorios y los fármacos utilizados para el tratamiento del colesterol fueron los más detectados. De nuevo, los valores más elevados de concentración correspondieron a los analgésicos/antiinflamatorios.

En la Figura 2.8 se muestran las concentraciones medianas de los fármacos estudiados en las tres depuradoras. Los datos corresponden a las muestras de efluente recogidas en los meses de junio 2008 y enero 2009. En la representación se han omitido los compuestos detectados a concentraciones inferiores al LOQ y los que no se detectaron en ninguna ocasión.

Si se realiza una comparación entre las tres depuradoras se observa que los valores de concentración fueron similares a excepción del ácido salícilico. Este compuesto tan sólo se detectó en el mes de enero en la depuradora de Benicassim, con una elevada concentración mediana (73 μg/L), y en la de Castellón a niveles de concentración mucho más bajos (próximos al LOQ). En las muestras pertenecientes a la depuradora de Castellón recogidas en el mes de junio los niveles detectados para algunos compuestos fueron significativamente más altos. Estos datos se comentarán en el último apartado.

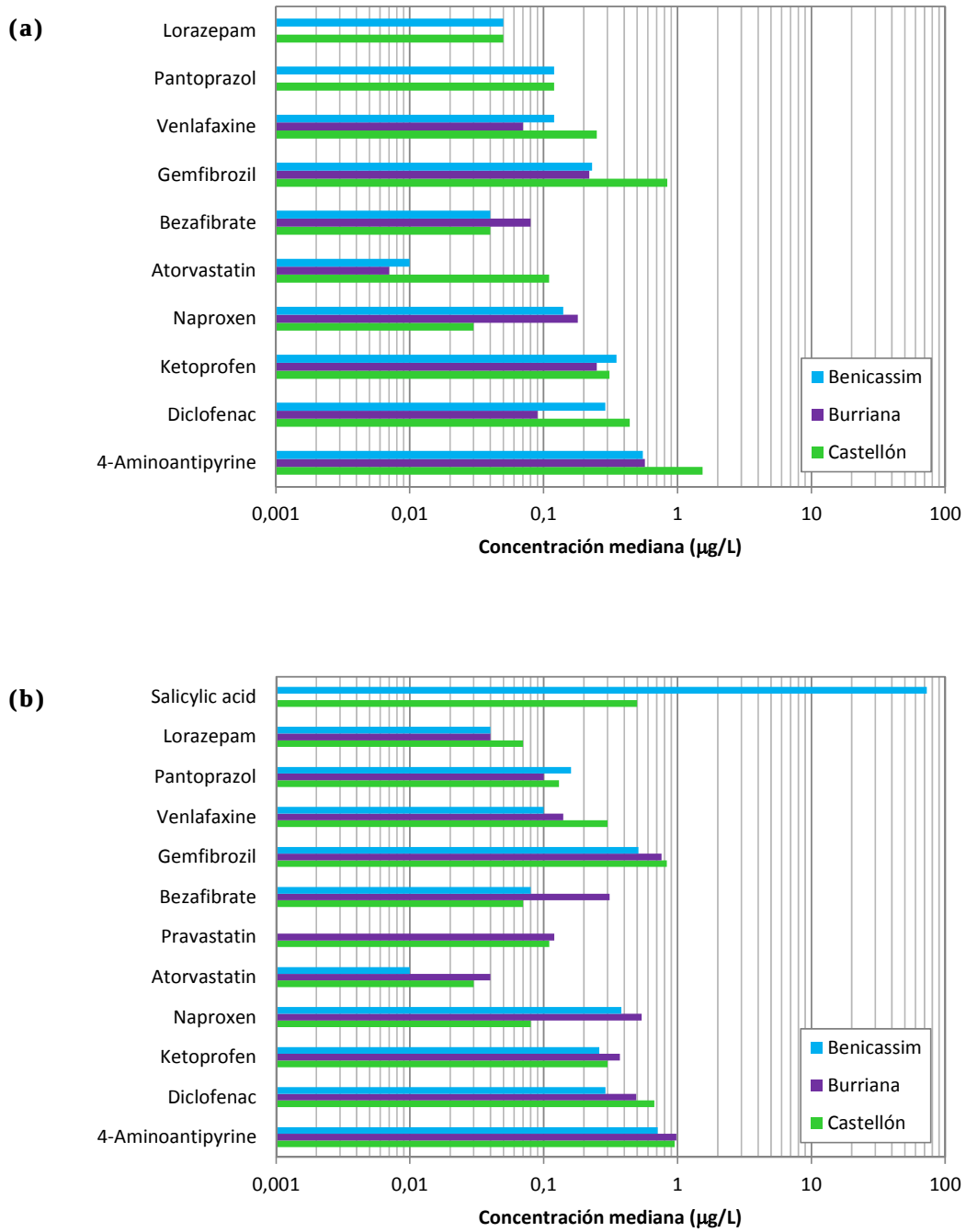

Figura 2.8 Concentración mediana (µg/L) de los fármacos encontrados en el efluente de tres EDAR. (a) Junio 2008. (b) Enero 2009.

Eliminación de fármacos durante el proceso de tratamiento

La eficacia de eliminación de una planta depuradora (RE) refleja su capacidad para eliminar los contaminantes orgánicos y evitar que éstos pasen al medio ambiente. Como se ha indicado en la Introducción de este artículo científico, generalmente se determina como el porcentaje de reducción de la concentración de un compuesto al pasar del influente al efluente. En este trabajo, se calculó del siguiente modo: RE = $(C_{influente} - C_{efluente})/C_{influente}$. Un valor de RE positivo refleja que tras el tratamiento de depuración, la concentración de un compuesto se ha reducido. Por el contrario, un valor negativo indica que su concentración es mayor en el efluente que en el influente.

Dos de los compuestos (acetaminofeno y enalapril) se eliminaron completamente durante el proceso de tratamiento. En el caso de compuestos como ibuprofeno, ácido salicílico, atorvastatina y pravastatina, se produjo una reducción del porcentaje de las muestras positivas y una disminución de los niveles de concentración detectados en el efluente. Numerosos fármacos, aunque su concentración se redujo tras el proceso de depuración, se detectaron en todas las muestras de efluente analizadas. Este fue el caso de la venlafaxina así como de la mayoría de los analgésicos/antiinflamatorios y de los fármacos empleados para el tratamiento del colesterol. Se trata de compuestos que parecen ser persistentes al tratamiento de depuración empleado (tratamiento convencional).

Cabe mencionar otro grupo de fármacos que no se detectaron en el influente pero sí en el efluente. Esta situación puede deberse a distintas causas: por un lado, en el caso de los compuestos con concentraciones muy bajas en el efluente (alrededor o incluso por debajo del nivel del LOQ), es posible que también estuvieran presentes en las muestras de influente. Sin embargo, la complejidad de esta matriz y la fuerte supresión de la ionización y de la señal que generalmente le acompaña, pudieron impedir su detección a niveles muy bajos de concentración. Por otro lado, también puede deberse a la conjugación. Los fármacos se metabolizan en mayor o menor medida en el hígado dando lugar a la formación de glucurónidos y otros compuestos conjugados. Cuando estos compuestos son sometidos al proceso de depuración podría producirse la liberación del fármaco (compuesto nativo), de modo que los niveles de

concentración aumentarían en el efluente. Esta última es la explicación más común en la literatura.

Como puede deducirse al observar las Figuras 2.7 y 2.8, la eficacia de eliminación de las tres depuradoras fue similar. Esto es lógico porque las condiciones operacionales que utilizan son también muy similares.

Segundo muestreo

Con el fin de tener una visión más amplia sobre la presencia de los fármacos en el medio ambiente la lista de compuestos analizados se amplió con un gran número de antibióticos. Así pues, se estudió la presencia de 47 compuestos en 28 muestras procedentes de la EDAR de Castellón de la Plana. Las muestras se recogieron en los meses de abril y octubre de 2009.

Muestras de influente

En el caso de los 20 fármacos seleccionados desde el inicio, los resultados obtenidos fueron similares a los del primer muestreo tanto en su frecuencia de detección como en las concentraciones encontradas. Únicamente el diclofenaco se detectó en un porcentaje menor de ocasiones.

Se encontraron nueve antibióticos, siete de los cuales estaban presentes en todas las muestras. De ellos, cuatro pertenecían al grupo de las quinolonas, destacando el ciprofloxacino.

En la Figura 2.9 se han representado las concentraciones medianas de los compuestos detectados en las muestras de influente analizadas en este muestreo.

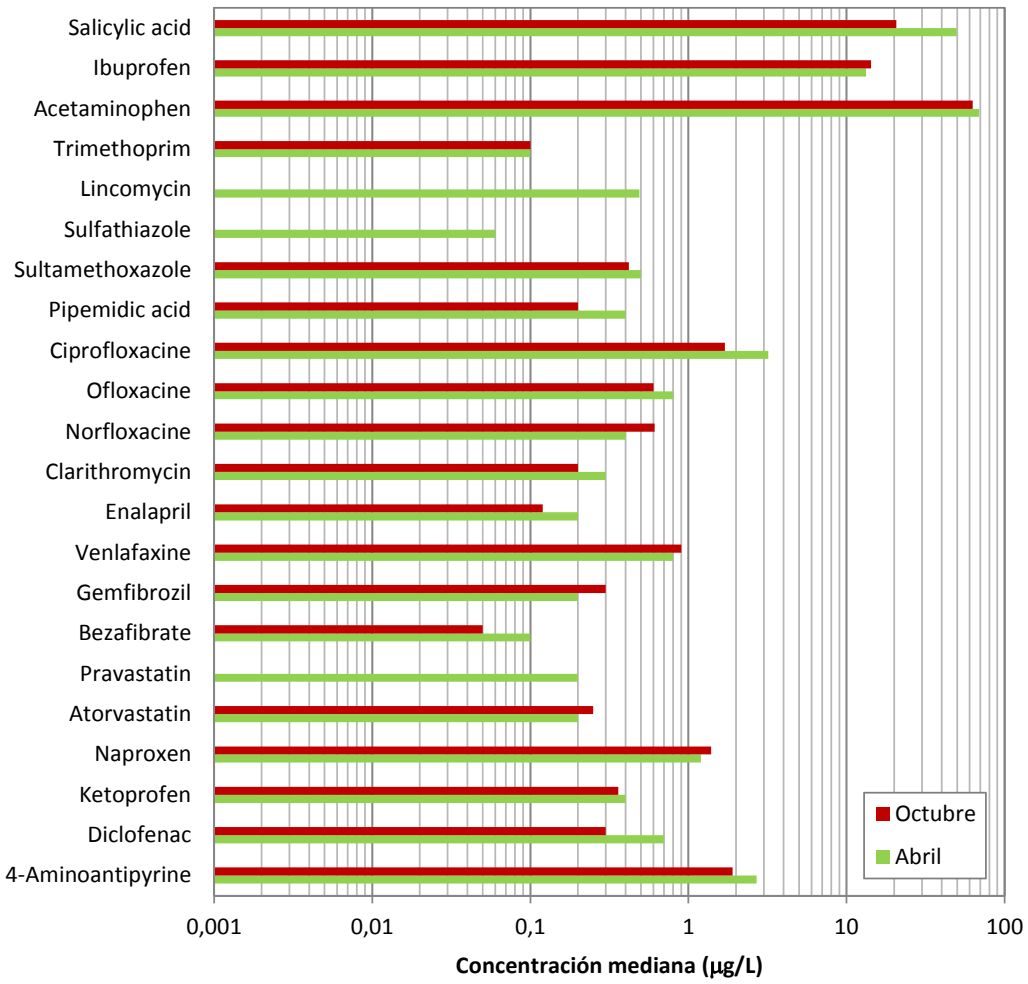

Figura 2.9 Concentración mediana (µg/L) de los fármacos encontrados en el influente de la depuradora de Castellón de la Plana en los meses de abril y octubre de 2009.

Muestras de efluente

Excepto 2 de los 23 fármacos hallados en las muestras, todos mostraron un porcentaje de detección superior al 90%. Los analgésicos/antiinflamatorios y el gemfibrozil (regulador lipídico) fueron los compuestos que se encontraron a mayor concentración, tal como se muestra en la Figura 2.10. Estos resultados son coherentes con los del primer muestreo. Tan sólo podríamos señalar diferencias en el ácido

salicílico que en el segundo muestreo no se detectó en ninguna ocasión frente al 26% detectado en el primero.

Las concentraciones de antibióticos fueron menores que las del resto de fármacos excepto en el caso de las quinolonas. Los niveles para este grupo de compuestos fueron comparables con los de los analgésicos/antiinflamatorios. Destacan las concentraciones del ciprofloxacino y ofloxacino superiores a los 0.6 y 0.4 µg/L respectivamente.

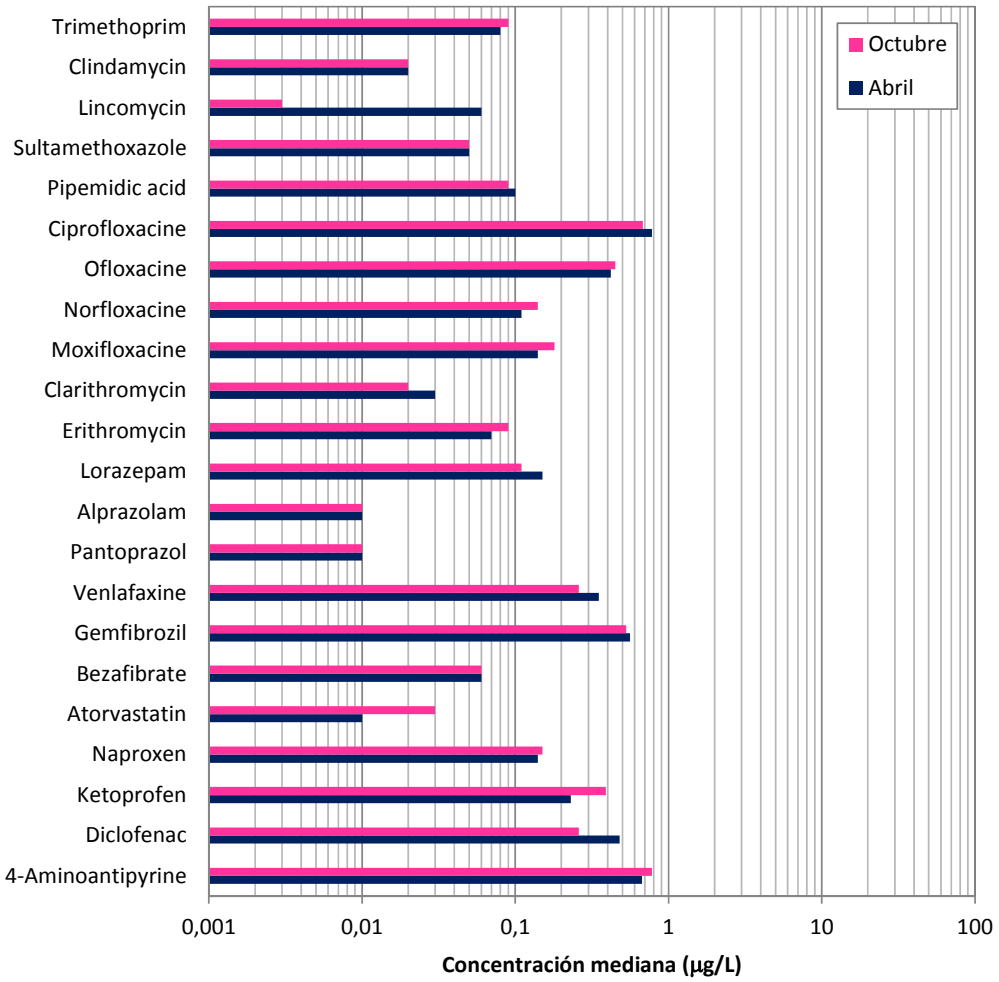

Figura 2.10 Concentración mediana (µg/L) de los fármacos encontrados en el efluente de la depuradora de Castellón de la Plana en los meses de abril y octubre de 2009.

Eliminación durante el proceso de tratamiento

Según el comportamiento de los fármacos observado en las EDAR podemos clasificarlos en varios grupos. En primer lugar, los compuestos que se eliminaron completamente en la planta depuradora. Este fue el caso del acetaminofeno, ibuprofeno, ácido salicílico y enalapril. Otros analitos se eliminaron parcialmente, es decir, tras el tratamiento de depuración empleado su concentración en el efluente disminuyó. Dentro de este grupo encontramos, entre otros, algunos fármacos empleados para el tratamiento del colesterol. Un tercer grupo estaría constituido por aquellos fármacos que únicamente se detectaron en las muestras de efluente. Se trata, por tanto, de compuestos con una eficacia de recuperación "negativa". Por último, distinguimos el caso de algunos compuestos que no se detectaron en ninguna muestra, ni de influente ni de efluente.

En la *Figura 3, artículo científico 3* se ha representado la eficacia de eliminación de la planta de depuración de Castellón. Atendiendo a la clasificación de los compuestos según su comportamiento en la EDAR, los fármacos pertenecientes al primer grupo son aquellos con valores de RE = 100 %. Los compuestos del segundo grupo presentan valores de RE positivos pero menores de 100%. Para poder representar el comportamiento del tercer grupo de compuestos se les asignó un valor arbitrario de -100%. Mención especial para el caso de tan sólo dos compuestos –gemfibrozil y diclofenaco- cuya concentración tras el proceso de depuración fue mayor que al inicio. Estos dos compuestos presentan valores de RE negativos. Su comportamiento, aunque puede resultar extraño, se ha observado también en otros estudios publicados.

Variación estacional

Por último, se realizó un estudio de la variación estacional de los niveles de fármacos en las aguas. Para ello se compararon los valores de concentración a lo largo del año en la depuradora de Castellón.

Para los 20 fármacos seleccionados desde el inicio, los valores de concentración fueron relativamente constantes. Únicamente para los compuestos pertenecientes al grupo de los analgésicos/antiinflamatorios se observó una mayor concentración en las muestras recogidas en invierno. Estos resultados son coherentes con su patrón de consumo ya que los analgésicos/antiinflamatorios se consumen durante todo el año pero especialmente en invierno. En cambio, el consumo de otros fármacos estudiados suele obedecer a tratamientos de larga duración, es decir, su uso no varía con los periodos estacionales. Este es, por ejemplo, el caso de los compuestos recetados para el tratamiento del colesterol.

Respecto a los antibióticos, el estudio de la variación estacional se realizó solamente en primavera y otoño. Las concentraciones obtenidas en las dos épocas del año fueron similares.

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2.5 Investigación de contaminantes emergentes, incluyendo productos de cuidado personal y fármacos, en aguas superficiales y efluentes urbanos

2.5.1 Introducción

Hoy en día resulta difícil encontrar en el mercado productos de higiene y cosmética que no contengan ingredientes químicos artificiales. En la fabricación de jabones, geles, dentífricos, desodorantes, cremas, agentes protectores solares, fragancias, lociones, etc. se utilizan alrededor de 7.000 sustancias diferentes, conocidas bajo el término de productos de cuidado personal (*personal care products*, PCPs). Algunos de estos compuestos también se emplean en la elaboración de alimentos y bebidas.

Son compuestos que se consumen a diario a nivel mundial en grandes cantidades (Ellis, 2006; Pedrouzo, 2011). A modo de ejemplo, en un estudio publicado en el año 1984 se estimaba que los parabenos, ya sea individualmente o combinados con otros ingredientes, se utilizaban en alrededor de 13.200 formulaciones en casi todos los tipos de cosméticos (Liebert, 1984). Es muy probable que en la actualidad esta cifra sea todavía mayor. Otro ejemplo que pone de manifiesto el elevado uso de este tipo de compuestos lo encontramos en los filtros UV orgánicos. Con el fin de proteger eficazmente frente a la radiación UV y para aumentar la estabilidad de los productos utilizados como filtros solares, generalmente a estos productos se les añaden entre tres y ocho filtros UV (Li, 2007).

Hasta hace pocos años la presencia de PCPs en el agua había pasado desapercibida por lo que, aunque se dispone de información sobre ellos, la bibliografía disponible es mucho menor que en el caso de los fármacos. Sin embargo, los posibles efectos secundarios que pueden ocasionar les han convertido en compuestos de interés.

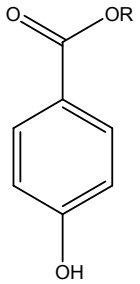
Los PCPs pueden llegar al medio ambiente en concentraciones que podrían resultar peligrosas para los organismos vivos. De hecho, presentan una toxicidad pronunciada en algas y microbios (Pedrouzo, 2009). Pero es sobre todo su posible capacidad para actuar como disruptores endocrinos lo que les ha convertido en objeto de estudio.

Entre las distintas clases de productos de cuidado personal, los preservantes y los filtros UV orgánicos resultan especialmente preocupantes.

Los filtros UV son compuestos lipofílicos y, por ello, pueden acumularse en el cuerpo humano y en el medio ambiente (Li, 2007). Algunos estudios indican que estos compuestos podrían tener actividad estrogénica y hormonal en peces (Fent, 2010). Por ese motivo no todos los compuestos pertenecientes a este grupo están permitidos, y para aquellos que pueden utilizarse, su concentración no puede superar los límites establecidos en la legislación. Por ejemplo, en la Unión Europea se ha autorizado el uso de 27 filtros UV, incluyendo la benzofenona-3 y la benzofenona-4. La concentración máxima autorizada para el primer compuesto en los productos utilizados como filtros solares es 10% en la UE (Directiva 83/574/CEE) mientras que en Estados Unidos (FDA, *Department of Health and Human Services*) y en Japón (*The Society of Japanese Pharmacopoeia*) la legislación es algo más restrictiva (6% y 5% respectivamente).

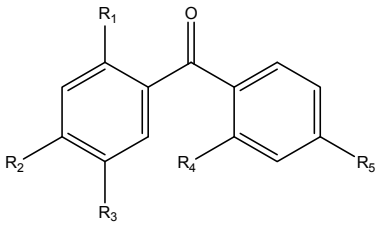
Los preservantes más utilizados en los productos de cuidado personal son los ésteres del ácido p-hidroxibenzoico (PHBA), más conocidos como parabenos. Estos compuestos poseen actividad estrogénica, la cual depende de su estructura química (Golden, 2005; Routledge, 1998; Jiménez-Díaz, 2011). Aunque esta actividad es menor en comparación con la de otros compuestos (ej. 17 β-estradiol), es preocupante porque los parabenos se encuentran en el medio ambiente en una concentración mucho mayor (González-Mariño, 2009). Además, la presencia de algunos parabenos en tejido de mama ha sugerido una posible relación entre estos compuestos y el cáncer de mama (Darbre, 2004). También se ha alertado sobre el peligro que podría suponer su uso en los desodorantes. Estos datos han provocado cierta preocupación sobre la seguridad de estos productos. Prueba de ello es que cada vez resulta más frecuente encontrar productos en el mercado, sobre todo cremas y cosméticos, en los que se destaca que carecen de parabenos.

En la Figura 2.11 se muestra la estructura general de los parabenos y de las benzofenonas.



Estructura general de un parabeno (ácido p-hidroxibenzoico)

R = *methyl* (CH_3), *ethyl* (C_2H_5), *propyl* (C_3H_7), *butyl* (C_4H_9)



Estructura general de una benzofenona

Benzophenone R_1:H; R_2:H; R_3: H; R_4: H; R_5: H

Benzophenone-1 R_1:OH; R_2:OH; R_3: H; R_4: H; R_5: H

Benzophenone-2 R_1:OH; R_2:OH; R_3: H; R_4: OH; R_5: OH

Benzophenone-3 R_1:OH; R_2:OCH_3; R_3: H; R_4: H; R_5: H

Benzophenone-4 R_1:OH; R_2:OCH_3; R_3: SO_3H; R_4: H; R_5: H

Figura 2.11 Estructura general de los parabenos y de las benzofenonas

La exposición a los PCPs puede producirse mediante inhalación, ingestión o contacto dérmico, siendo ésta última la principal. Tras su uso, los PCPs pueden llegar al medio ambiente acuático principalmente a través de dos vías. Una de ellas es la introducción directa a partir de actividades recreacionales, es decir, tras liberarse de la piel de las personas que se aplican productos cosméticos antes de bañarse en la piscina, ríos o mares, y otra es a través de las EDAR (introducción indirecta). En este último caso podrían producirse, por ejemplo, al lavar la ropa o las toallas que han estado en contacto con la piel o incluso mediante su excreción en la orina tras su aplicación y absorción cutánea u oral (barras de labios) (Li, 2007). En cualquier caso, su presencia en ríos y en aguas de EDAR se ha confirmado en todo el mundo (Pedrouzo, 2011).

Muchas veces los PCPs se analizan junto con los fármacos y se les cataloga como un único grupo llamado PPCPs, que corresponde a las siglas en inglés *pharmaceuticals and personal care products.*

En el presente artículo científico se seleccionaron 17 PPCPs. En el caso de los fármacos, su elección se basó en su elevado consumo (diclofenaco, ibuprofeno y ácido clofíbrico), así como cinco fármacos más que fueron seleccionados por haberse detectado en las aguas de Castellón mediante *screening* con LC-QTOF MS en nuestro propio grupo de investigación (Díaz, 2012). Como se ha comentado en el Capítulo 1, el uso de estos analizadores en el análisis cuantitativo está aún limitado, mientras que los analizadores de triple cuadrupolo resultan ideales para dicho fin.

En cuanto a los productos de cuidado personal, se seleccionaron nueve compuestos para los que existe mayor preocupación ambiental, ya sea por su presencia frecuente o por sus efectos nocivos (posible actividad estrogénica).

Para todos ellos se realizó un estudio detallado del efecto matriz en diez muestras de agua distintas y se evaluó la capacidad de corrección de cinco analitos marcados isotópicamente usados como patrones internos.

Una de las novedades de este trabajo respecto a los publicados previamente es que el método desarrollado se validó en un total de diez muestras de agua (cinco aguas superficiales y cinco de efluente) fortificadas a dos niveles de concentración. El objetivo al utilizar este número de muestras, mucho mayor que el habitual en la mayoría de los métodos validados, es tener una visión más realista sobre el comportamiento y robustez del método en diferentes muestras de agua.

La metodología desarrollada se aplicó al análisis de 73 muestras de agua tomadas en distintos puntos de España y Colombia. Cabe destacar que este trabajo ha sido el primero en el que se publican datos sobre la presencia de PPCPs en Colombia.

2.5.2 Artículo científico 4

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Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high performance liquid-chromatography-tandem mass spectrometry

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ABSTRACT

In this work, a multi-class method for the simultaneous determination of 17 emerging contaminants, including pharmaceuticals and personal care products, has been developed. Target analytes were two anti-inflammatories, a lipid regulator agent, two angiotensin II antagonists, two antiepileptic drugs and a diuretic. Among personal care products, four preservatives and five UV filters were included. The method is based on solid-phase extraction (SPE) using Oasis HLB cartridges followed by ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Up to three simultaneous transitions per compound were acquired to assure a reliable identification. A detailed study of the extraction process efficiency and matrix effects was carried out in surface water and effluent wastewater. The use of isotope-labeled internal standards (ILIS) was tested to compensate both potential SPE losses during sample extraction and signal suppression/enhancement observed, especially in EWW. Satisfactory correction in all water samples was only ensured when the own analyte ILIS was used. The use of analogues ILIS was a rather useful approach for correction in the majority of the samples tested when analyte ILIS was unavailable. The method was successfully validated in five different surface water (SW) samples and five effluent wastewater (EWW) samples spiked at two concentration levels (0.05 and 0.5 μg/L in SW; 0.1 and 0.5 μg/L in EWW). The developed method was applied to the analysis of 22 samples (SW and EWW) from the Spanish Mediterranean area and 51 reservoir water samples from Colombia. Personal care products were frequently detected, with the highest concentrations corresponding to benzophenone and benzophenone-4 (samples from Spain), and methylparaben (samples from Colombia). Several pharmaceuticals were detected in the Spanish samples, where irbesartan and valsartan – two Angiotensin II antagonists that are not commonly monitored in the aquatic environment – were the compounds most frequently detected.

1. Introduction

Pharmaceuticals and personal care products (PPCPs) are a diverse group of chemicals that include human and veterinary drugs, food supplements and other chemicals used in cosmetics, fragrances and sun-screen agents [1]. They have become the focus of global environmental researchers' attention over the last decade [2] due to their extensive human and veterinary consumption and their potential negative impact on the environment, living organisms and human health. PPCPs are suspected to cause elevated rates of cancer, reproductive impairment in humans and other animals and development and spread of antimicrobial resistance [2–4]. Recent studies reported that these contaminants do not need to be persistent in the environment to cause negative effects due to their continuous release [1,5,6]. They have been detected in different natural water systems that include rivers, lakes and reservoirs [7–9].

PPCPs enter into the aquatic environment mainly through effluents of wastewater treatment plants (WWTPs) [6]. A few compounds are transformed or retained in the sludge by conventional water treatment processes. However, most of compounds are persistent and polar, and survive the passage through WWTPs, being released via effluents into receiving aquatic environments [1–3].

Pharmaceuticals have been extensively studied in the last years due to their large consumption and environmental implications. However, until recently, less interest has been paid to the presence of personal care products (PCPs) in environmental waters [10]. PCPs include UV-filters, preservatives, antimicrobials, musk fragrances, insect repellents and, in general, ingredients or excipients

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validated in five different surface water (SW) samples and five effluent wastewater (EWW) samples spiked at two concentration levels (0.05 and 0.5 μg/L in SW; 0.1 and 0.5 μg/L in EWW). The developed method was applied to the analysis of 22 samples (SW and EWW) from the Spanish Mediterranean area and 51 reservoir water samples from Colombia. Personal care products were frequently detected, with the highest concentrations corresponding to benzophenone and benzophenone-4 (samples from Spain), and methylparaben (samples from Colombia). Several pharmaceuticals were detected in the Spanish samples, where irbesartan and valsartan – two Angiotensin II antagonists that are not commonly monitored in the aquatic environment – were the compounds most frequently detected.

Keywords

Pharmaceuticals; Personal care products; Ultra-high performance liquid chromatography; Tandem mass spectrometry; Matrix effects; Surface water and wastewater.

1. Introduction

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Pharmaceuticals have been extensively studied in the last years due to their large consumption and environmental implications. However, until recently, less interest has been

paid to the presence of personal care products (PCPs) in environmental waters [10]. PCPs include UV-filters, preservatives, antimicrobials, musk fragrances, insect repellents and, in general, ingredients or excipients used in cosmetics, food supplements, shampoos, toothpastes, sun screens agents, antiseptics and personal care products formulations and manufacture [1, 10]. Among them, UV filters and preservatives are considered of higher relevance. UV filters are used as ingredients in some sunscreens and a wide variety of cosmetics (skin and hair care products, lotions, creams, fragrances) [11-13]. These compounds enter the aquatic environment either directly via wash-off from skin and cloth during recreational activities, or indirectly via wastewater or swimming pool waters [11]. The most commonly UV filters are benzophenones. Two of them, benzophenone-3 and benzophenone-4 are included in the list of compounds that can be employed as UV filters in sunscreen cosmetic products according to the European Union Legislation [14]. Benzophenone, benzophenone-1 and benzophenone-2 seem to present hormonal activity [11-12]. Moreover, benzophenone has been listed among chemicals suspected of having endocrine disrupting effects [12]. These compounds are characterised by the presence of aromatic rings, often with attached hydrophobic groups. They are lipophilic and can therefore be accumulated in the human body and the environment [15].

Among the group of preservatives, parabens are the most commonly used. These are esters of p-hydroxybenzoic acid and are suspected to be endocrine disruptors [10, 16] Parabens are widely used, due to their anti-bactericidal and anti-fungicidal properties, in cosmetic products and pharmaceuticals, and in food and beverage processing [16-18].

Advanced analytical methodology is needed in order to study the occurrence and distribution of PPCPs in the environment. Most of recent methods are based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) using triple quadrupole (QqQ) analyzer [10], which has become the technique of choice in this field due to its high selectivity and sensitivity [2]. However, one of the major drawbacks associated to LC-MS/MS methods is matrix effect, which results in suppression or enhancement of the analyte signal due to the presence of co-extracted matrix components that affect analyte ionization. These undesirable effects can hamper the identification, and drastically affect the quantification of analytes, especially when dealing with complex matrices [19].

The goal of this paper is to develop rapid, accurate and sensitive analytical methodology based on UHPLC–MS/MS for the simultaneous determination (quantification and confirmation) of PPCPs in surface and wastewater. A notable number of PPCPs (17 compounds) has been included in the method scope in order to have wider and more realistic

knowledge on the presence of these compounds in the environment. The main benzophenones, including those listed in the EU legislation, and the parabens most extensively used in cosmetics and food processing, methyl-, ethyl- and buthyl-paraben [10] have been included in the target list of analytes. Several ILIS have been tested for correction of matrix effects and/or potential losses associated to the SPE step. The method was applied to the analysis of 73 samples (surface water, effluent wastewater and reservoir water) collected at different sites in Spain and Colombia. Although emerging contaminants are still unregulated in water, their presence is a matter of concern, mainly in those cases where treated water is used for drinking water supply. This occurs in Colombia, where artificial reservoirs are frequently used to this aim. Reservoirs are supplied with water from rivers or tributaries, which receive urban and agricultural wastewater discharges. For this reason, it is important to monitor water quality in Colombia's reservoirs, specifically for those PPCPs used in human applications and households. The presence and distribution of PPCPs in Colombia have not been reported, and to the best of our knowledge, this is the first study reporting data on PPCPs in this country. The methodology developed in this work will be implemented in the laboratory of GDCON group from Antioquia University (Colombia) since 2012.

2. Experimental

2.1. Reagents and chemicals

Reference standards were purchased from Sigma-Aldrich (St Louis, MO, USA), LGC Promochem (London, UK), Dr. Ehrenstorfer (Augsburg, Germany) and Toronto Research Chemicals (Ontario, Canada). Their chemical structure is shown in Fig. 1.

Isotopically labeled compounds diclofenac-d_4, ibuprofen-d_3, valsartan-d_8, ethylparaben-d_4 and benzophenone-3-d_5, were from CDN Isotopes (Quebec, Canada).

HPLC-grade methanol (MeOH) and HPLC-grade acetonitrile (ACN) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water was obtained from distilled water passed through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac, reagent grade) and ammonia (NH_3, solution 32%, reagent grade) were supplied by Scharlab (Barcelona, Spain).

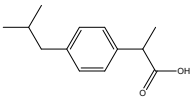
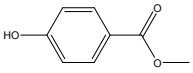
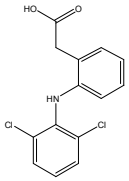
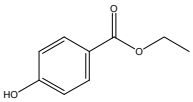
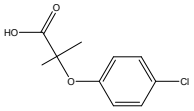
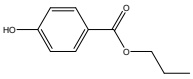
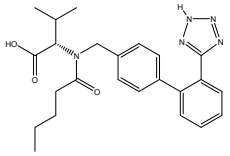
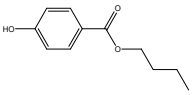
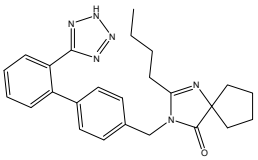
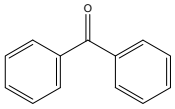
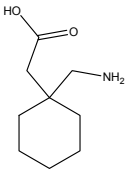
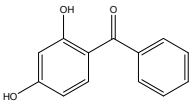
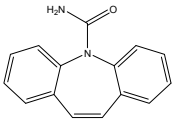
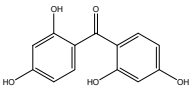
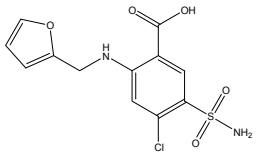
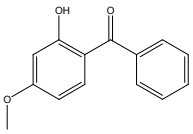
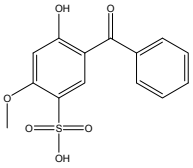
Compound	Structure	Compound	Structure
Ibuprofen $C_{13}H_{18}O_2$		Methylparaben $C_8H_8O_3$	
Diclofenac $C_{14}H_{11}NCl_2O_2$		Ethylparaben $C_9H_{10}O_3$	
Clofibric Acid $C_{10}H_{11}ClO_3$		Propylparaben $C_{10}H_{12}O_3$	
Valsartan $C_{24}H_{29}N_5O_3$		Butylparaben $C_{11}H_{14}O_3$	
Irbesartan $C_{25}H_{28}N_6O$		Benzophenone $C_{11}H_{14}O_3$	
Gabapentin $C_9H_{17}NO_2$		Benzophenone-1 $C_{13}H_{10}O_3$	
Carbamazepine $C_{15}H_{12}N_2O$		Benzophenone-2 $C_{13}H_{10}O_5$	
Furosemide $C_{12}H_{11}ClN_2O_5S$		Benzophenone-3 $C_{14}H_{12}O_{3c}$	
		Benzophenone-4 $C_{14}H_{12}O_6S$	

Fig. 1. Structures and molecular formula of the selected compounds.

Individual stock solutions of PPCPs were prepared dissolving 25 mg, accurately weighted, in 50 mL methanol, obtaining a final concentration of 500 mg/L. Intermediate solutions were prepared in methanol, and were used to prepare a mix of all compounds at 500 µg/L in methanol–water (40:60, v/v). This mix solution was subsequently diluted with HPLC-grade water to prepare working solutions.

Individual stock solutions of ILIS were prepared in methanol. A mix working solution at 250 µg/L was prepared in water and used as surrogate.

All standard solutions were stored in amber glass bottles. Stock solutions were stored at −20 °C, and intermediate and working solutions at 4 °C.

Cartridges used for SPE were Oasis HLB (60 mg) and Oasis MCX (150 mg) from Waters (Milford, MA, USA).

2.2. Liquid chromatography

UHPLC analysis were carried out using an Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was performed using an Acquity UPLC BEH C18 column, 1.7 µm, 50 mm×2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 40 °C and the sample manager was maintained at 5 °C. Mobile phase consisted of a water 0.01% HCOOH/methanol gradient. The methanol percentage was changed linearly as follows: 0 min, 10%; 9 min, 90%; 10 min, 90%; 10.1 min; 10%. Analysis run time was 12 min. The sample injection volume was 20 µL.

2.3. Mass spectrometry

A TQD (triple quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray (ESI) was used (Waters Corp., Milford, MA, USA). Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N_2 LC–MS (Claind, Teknokroma, Barcelona, Spain). The cone gas and the desolvation gas flows were set at 60 and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) at 2×10^{-3} mbar in the T-Wave collision cell. Capillary voltages of −3.0 kV (negative ionization

mode) and 3.5 kV (positive ionization mode) were applied. The interface temperature was set to 500 °C and the source temperature to 120 °C. A dwell time of 0.01 s was selected.

Masslynx 4.1 (Micromass, Manchester, UK) software was used to process quantitative data.

2.4. Recommended procedure

Water samples were centrifuged at 4500 rpm for 5 min, when suspended particulate matter was observed. 100 mL water sample were taken and 100 µL of the ILIS mix working solution were added to give a final concentration for each ILIS of 0.25 µg/L. Oasis HLB cartridges were conditioned with 3 mL MeOH and 3 mL HPLC-grade water before use. Then, the samples were loaded onto the cartridge and, after drying under vacuum, analytes were eluted with 5 mL methanol. The extract was evaporated to dryness under a gentle nitrogen stream at 40 °C and reconstituted with 1 mL MeOH–water (10:90, v/v). Finally, 20 µL were injected in the UHPLC–MS/MS system under the conditions shown in Table 1. Quantification was made with calibration standards prepared in solvent, using relative responses analyte/ILIS, or absolute responses, depending on whether ILIS was used for correction or not. ILIS were used to correct for matrix effects and/or SPE potential errors as shown in Tables 2 and 3.

2.5. Validation study

The linearity of the method was studied by analyzing standard solutions (in triplicate) at seven concentrations in the range from 1 to 100 µg/L (equivalent to 0.01–1 µg/L in the water sample). Satisfactory linearity using least squares regression was assumed when the correlation coefficient (*r*) was higher than 0.99 and residuals lower than 30% without significant trend, based on relative responses (analyte peak area/ILIS peak area), except for those compounds that were quantified without ILIS (absolute response).

Accuracy (expressed as percentage recovery) and precision (expressed in terms of relative standard deviation (RSD)) were studied by means of recovery experiments in several surface water (SW) and effluent wastewater (EWW) spiked at two concentrations (0.05 in 0.5 µg/L in SW; 0.1 and 0.5 µg/L in EWW). The method was validated in five SW samples collected in different sites of the Mediterranean Spanish area of Valencia (Mijares and Jucar rivers, and

Albufera de Valencia, Clot de Burriana, and Tavernes de la Valldigna lakes) and five 24-h composite EWW samples collected from the Castellón de la Plana WWTP along five consecutive days. For each individual sample, recovery experiments were performed in duplicate, giving a total number of 10 data for SW and 10 for EWW at each spiked concentration.

The limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. True blank samples were not found for several analytes, which were present in the samples tested. In these cases, LOQ values were estimated from the analyte levels quantified in the non-spiked "blanks". The instrumental limit of detection (LOD) was estimated for *S*/*N*=3 from the chromatograms of standards at the lowest concentration level.

2.6. Application to water samples

The method was applied to 73 samples (surface water, effluent wastewater and reservoir water) collected at different sites in Spain and Colombia.

Eleven SW samples were collected at selected sites from the Spanish Mediterranean area of Valencia. Another eleven EWW were also collected, consisting on 24-h composite urban wastewater samples, from different WWTPs of the same area. All samples were stored in the dark at −18 °C in polyethylene high-density bottles until analysis.

Fifty-one reservoir water samples were collected from two reservoirs situated in the department of Antioquia (Colombia) that are used for drinking water supply. In total, eleven sampling locations were selected for reservoir 1 (located in the east of Antioquia), and 10 for reservoir 2 (in the north of Antioquia) and collected at three depths in the water column: subsurface, photic zone limit and reservoir bottom. Due to the low depth, it was only possible to take samples at the surface and at the bottom in some stations. Samples were collected in July and August 2011 using a Schindler bottle. Then, 100 mL of each sample were transferred to a polyethylene bottle. A total of 51 samples were collected and transported to the laboratory under cooled conditions (4 °C). Upon reception in the Colombian laboratory, samples were immediately processed (SPE) and the cartridges were sent to Spain for LC–MS/MS analysis. No stability test of analytes on the SPE cartridges was made.

3. Results and discussion

In this work, 17 pharmaceuticals and personal care products (PPCPs) were studied. Pharmaceuticals selected belong to different therapeutical groups: anti-inflammatories, antiepileptic drugs, lipid regulators, angiotensin II antagonists and diuretics. The choice of diclofenac, ibuprofen and clofibric acid was based on their high usage in both Spain and Colombia [20]. Valsartan, irbesartan, gabapentin, carbamazepine and furosemide have been frequently detected in wastewater of the Valencian area [21]. The remaining nine compounds were personal care products (UV filters and preservatives). They were selected due to their wide use and to their potential harmful effects on human and aquatic organisms [10, 22].

3.1. MS and MS/MS optimization

Full-scan and MS/MS mass spectra of analytes were obtained from infusion of 1 mg/L methanol/water (50:50, v/v) individual standard solutions at a flow rate of 10 μL/min. For those compounds that showed abundant sodium adduct $[M+Na]^+$ (e.g. carbamazepine), the addition of HCOOH into the infusion vial favored the $[M+H]^+$ formation. NH_4Ac was also added into the infusion vial in order to evaluate the possible presence of ammonium adducts, or to minimize the $[M+Na]^+$ formation. Although sodium adducts decreased in presence of ammonium, the abundance of the protonated molecules was lower than using formic acid. Therefore, formic acid was selected as additive.

Valsartan, irbesartan, benzophenone-1, benzophenone-2 and benzophenone-3 were ionized in both positive and negative modes. With the exception of benzophenone-2, positive ionization mode was selected because of the better sensitivity reached under this mode. The most abundant ion was $[M+H]^+$, or $[M–H]^–$, and it was selected as precursor ion. The presence of halogenated atoms (Cl) in some compounds (diclofenac and clofibric acid) allowed using two different precursor ions (corresponding to ^{35}Cl and ^{37}Cl, respectively).

Three SRM transitions were selected for each compound to assure the reliable confirmation of the compound detected. The most abundant product ion was used for quantification (*Q*) whereas the second and the third most sensitive transitions were used for confirmation (q_1 and q_2). For ibuprofen, diclofenac, irbesartan, parabens and some benzophenones, only one (ibuprofen) or two transitions (the rest of compounds) could be monitored due to their poor fragmentation. For example, for parabens the *m*/*z*=92 product ion,

corresponding to $[C_6H_4O]^-$, was selected. This ion was the most abundant fragment for methylparaben, ethylparaben and propylparaben, and therefore it was selected for quantification, while for butylparaben it was selected for confirmation. For this compound, $[M–H–CH_2CH_2CH_2CH_3]^-$, corresponding to the lost of a butyl radical, was the most abundant ion [22, 23].

For benzophenone-1 and benzophenone-3, similar fragment ions were obtained due to their analogous chemical structure. In both cases, the loss of the benzene group $[M+H-C_6H_6]^+$ corresponded to the main product ion (selected for quantification), while $[C_7H_5O]^+$ was selected for confirmation, as previously reported [24].

Regarding ILIS, only one transition was required. In the case of diclofenac-d_4, the transition 300.1>256.1 was chosen in order to avoid the overlap between the natural analyte (isotope peak due to the presence of two chlorine atoms; $2Cl^{37}$) and the ILIS signal (d_4), which would have occurred if the transition 298.1>254.1 had been chosen. In order to obtain enough points to define the chromatographic peak, the SRM transitions were distributed along six overlapping windows using dwell times of 10 ms. This favorable overlapping between positive and negative time windows was possible due to the low positive-to-negative-switching time (20 ms) attainable by the triple quadrupole analyzer used in this work.

Mass spectrometry parameters, precursor and product ions selected, instrumental LODs and ion ratios (Q/q) used for confirmation are shown in Table 1.

Table 1
MS/MS optimized conditions for selected compounds.

Compound	Group	Polarity (ES)	LOD (pg)	MW	Log Kow	Q transition	Cone (V)	C.E. (eV)	q_1 transition	C.E. (eV)	q_2 transition	C.E. (eV)	Q/q_1	$Q/q2$
Ibuprofen	Anti-inflammatories	-	107	206.1	4.0	205.1 > 161.1	30	10	-	-	-	-	-	-
Diclofenac		-	9.6	295.0	4.5	294.1 > 250.1	30	10	296.1 > 252.1	30	-	-	1.4	-
Clofibric acid	Lipid regulators	-	3.6	214.1	2.6	213.7 > 127.0	20	15	213.7 > 85.0	10	215.2 > 129.0	10	7.3	25.8
Valsartan	Angiotensin II antagonists	+	1.5	435.2	5.2	436.5 > 235.2	20	15	436.5 > 291.3	15	436.5 > 418.4	10	1.0	1.8
Irbesartan		+	0.3	428.2	5.3	429.5 > 195.3	30	20	429.5 > 207.3	25	-	-	4.1	-
Gabapentin	Antiepileptic drugs	+	1.4	171.1	- 1.1	172.3 > 154.3	25	15	172.3 > 137.2	15	154.2 > 55.1	20	2.3	4.6
Carbamazepine		+	0.5	236.1	2.5	237.3 > 194.2	25	25	237.3 > 179.2	35	237.3 > 165.2	40	4.9	5.6
Furosemide	Diuretics	-	2.7	330.0	2.3	329.2 > 205.1	30	20	329.2 > 285.2	15	329.2 > 77.9	30	1.1	3.8
Methyparaben	Preservatives	-	7.5	152.1	2	151.1 > 92.0	30	20	151.1 > 136.1	10	-	-	2.3	-
Ethylparaben		-	3.7	166.1	2.5	165.2 > 92.0	20	20	165.2 > 136.1	15	-	-	2.9	-
Propylparaben		-	1.8	180.1	3.0	179.2 > 92.0	30	20	179.2 > 136.1	20	-	-	2.8	-
Butylparaben		-	3.8	194.1	3.6	193.2 > 136.1	35	15	193.2 > 92.0	30	-	-	2.5	-
Benzophenone	UV filters	+	2.2	182.1	3.2	183.3 > 105.0	25	15	183.3 > 77.1	20	-	-	2.5	-
Benzophenone-1		+	10.6	214.0	3.0	215.3 > 137.1	25	20	215.3 > 105.1	20	-	-	7.5	-
Benzophenone-2		-	4.7	246.0	2.8	245.3 > 135.0	25	15	245.3 > 109.0	20	-	-	1.6	-
Benzophenone-3		+	2.6	228.1	3.8	229.3 > 151.1	25	20	229.3 > 105.1	20	229.3 > 77.1	35	2.4	2.6
Benzophenone-4		-	4.0	308.0	0.4	307.3 > 211.2	40	40	307.3 > 79.9	35	307.3 > 291.2	20	1.1	1.7
Ibuprofen-d_3		-	-	209.2	-	208.2 > 164.2	20	10	-	-	-	-	-	-
Diclofenac-d_4		-	-	299.1	-	300.1 > 256.1	30	10	-	-	-	-	-	-
Valsartan-d_8		+	-	443.3	-	444.4 > 291.2	20	20	-	-	-	-	-	-
Ethylparaben-d_4		-	-	170.1	-	169.2 > 96.1	25	25	-	-	-	-	-	-
Benzophenone-3-d_5		+	-	233.3	-	234.3 > 151.1	25	20	-	-	-	-	-	-

Abbreviations: ES, electrospray ionization; MW, monoisotopic molecular weight; Q, quantification; q, confirmation, C.E., collision energy.

3.2. Chromatographic conditions

In this work, a UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) was chosen. To optimize the chromatographic separation, methanol and acetonitrile were evaluated as mobile phase organic solvents, and HCOOH at various concentrations was tested. Acetonitrile was discarded because sensitivity got worse for parabens and for most compounds ionized under positive mode.

The use of solvents without any additive provided better sensitivity for most target compounds. However, poor peak shape was observed for several compounds (e.g. valsartan, benzophenone-2), which was improved when adding HCOOH.

The addition of HCOOH improved the chromatographic separation of several compounds determined in positive mode. The presence of acid also favored the chromatographic retention of acidic compounds, especially for diclofenac and clofibric acid, determined in negative mode. Finally, 0.01% HCOOH as the aqueous phase, and MeOH as organic phase, were selected as a compromise for the simultaneous chromatographic separation of both positive and negative ionized analytes.

3.3. Solid-phase extraction (SPE) study

Two cartridges with different retention mechanisms were compared in this work: the hydrophilic–lipophilic balanced Oasis HLB and the mixed polymeric-cation exchange sorbent Oasis MCX. Oasis HLB was tested at natural sample pH, while MCX cartridges required acidification of the water sample before loading in order to retain the protonated basic compounds under these conditions.

The SPE process efficiency for the selected compounds in both cartridges was tested in SW and EWW matrices. It was estimated as recovery percentage (RE).

For this purpose, the quotient between the responses obtained for samples spiked (0.5 μg/L) before SPE and for sample extracts spiked (50 μg/L) after SPE were compared [25]. This experiment was made in duplicate.

“Blank” samples, spiked only with the ILIS mix, were also processed to subtract the responses of possible target compounds that were normally present in the water samples.

The recoveries of the SPE process in both cartridges are depicted in Fig. 2 for a SW sample (Mijares river). Similar pattern was observed in EWW, although with slightly lower recoveries, surely due to the presence of matrix interferents, more abundant in wastewater than in surface water, resulting in a reduction of the sorption efficiency of cartridges.

In the case of Oasis HLB, satisfactory RE were obtained for all compounds except for gabapentin, benzophenone and benzophenone-3, which suffered some losses in the SPE process, especially significant for gabapentin. This analyte is small, highly polar molecule, with acidic and basic groups, and it is poorly retained on Oasis HLB. On the contrary, gabapentin was efficiently retained on Oasis MCX due to protonation of the free basic amino group, yielding higher recovery (up to 90%). In general, quantitative recoveries were observed for the majority of PPCPs in both cartridges, except for several parabens and benzophenones, which recoveries decreased considerably with MCX. As better recoveries for a higher number of compounds were achieved with Oasis HLB, these cartridges were selected for method development and validation purposes.

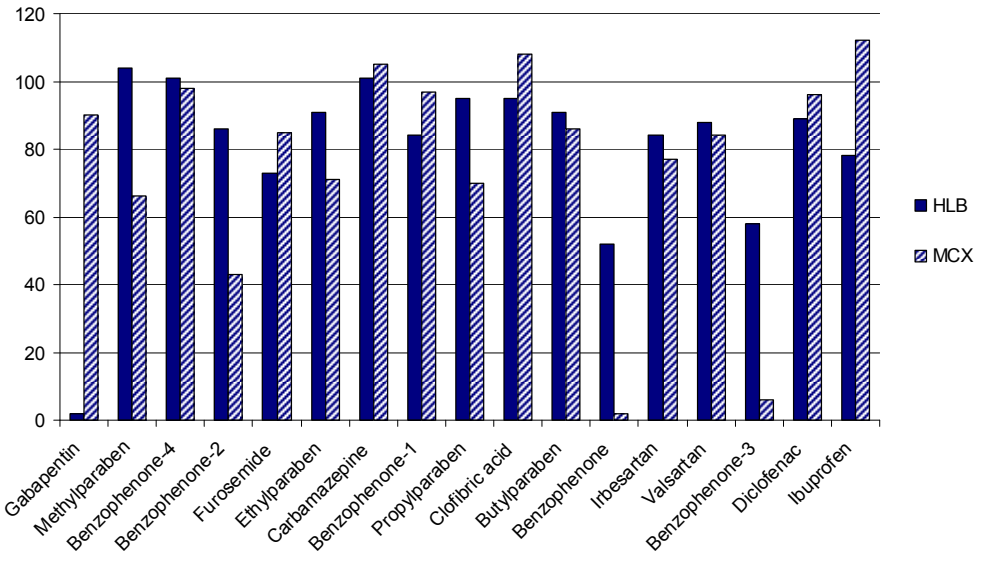


Fig. 2. Recoveries of the SPE process (RE) with Oasis HLB and Oasis MCX cartridges in SW sample.

The lower SPE efficiency for benzophenone could be corrected by the use of benzophenone-3-d_5, used as surrogate ILIS, in both SW and EWW. For the rest of compounds,

although it was not strictly necessary to correct the SPE losses, the use of ILIS slightly improved their extraction process efficiency but were mostly needed for matrix effects correction, as discussed below. The only exception was gabapentin, which low recoveries could not be corrected by any of the available ILIS due to its singular physico-chemical characteristics.

3.4. Matrix effect study

Matrix effect is one of the main factors affecting accuracy in LC–MS/MS methods. Matrix effects are due to the presence of coeluting matrix compounds that affect analyte ionization leading to notable errors in quantification, unless adequately corrected or minimized. Matrix effects correction is complicated in environmental analysis, where matrix-matched calibration approach is not easy to be applied. The use of ILIS is surely the most suitable way although difficult to apply in multi-residue methods where a high number of ILIS would be required for a reliable correction. In this work, a detailed study of matrix effects was made in a notable number of water samples. To this aim, 10 different samples were chosen (five SW and five EWW) and the use of several ILIS was evaluated.

The water extracts obtained after SPE were spiked at 50 µg/L for each individual compound, and the ILIS mix at 25 µg/L was also added. For each compound, the ratio between its response in the water extract and the response of the standard in solvent at the same concentration (i.e. 50 µg/L) was taken as matrix effect (ME) [25].

As expected, matrix effects in EWW were notably higher than in SW (Fig. 3) Signal suppression was observed for most PPCPs, although six of them showed ionization enhancement in both matrices (gabapentin, carbamapezine, benzophenone, benzophenone-1, valsartan and benzophenone-3). Only two compounds (benzophenone-4 and irbesartan) did not require correction in any of the samples tested. Consequently, matrix effect correction was necessary for the wide majority of PPCPs to obtain satisfactory results. In this work, the use of five ILIS was evaluated. As expected, when the analyte ILIS was available, the correction was highly satisfactory (i.e. ethylparaben, benzophenone-3, valsartan, diclofenac and ibuprofen) in all the water samples tested. For the remaining compounds, the selection of ILIS was based on the similarity in their chemical structure with the analytes under study. Thus, ethylparaben-d_4 was used to correct the paraben compounds and benzophenone-3-d_5 was used for benzophenones ionized in positive mode. However, for the rest of analytes the selection of an analogue ILIS was more problematic. The best approach seemed to be the use of an ILIS with

close retention time. For instance, furosemide was satisfactory corrected by ethylparaben-d_4 in SW (Fig. 3a); this ILIS was also able to compensate the ionization suppression suffered by this compound in EWW (Fig. 3b). In the case of clofibric acid, two different ILIS, both ionized under negative mode (ethylparaben-d_4 and diclofenac-d_4) could be selected. In SW, matrix effects correction was more satisfactory with ethylparaben-d_4 but it led to undesirable enhancement when used in EWW (data not shown). On the contrary, the use of diclofenac-d_4 compensated matrix effects from 50% up to around 100%, so this ILIS was chosen in EWW. Similar situation was observed for carbamazepine and benzophenone-1, where benzophenone-3-d_5 was able to correct matrix effects for both compounds in SW, whilst valsartan-d_8 was found to be more suitable for their quantification in EWW.

These examples illustrate that matrix effects are both compound- and matrix-dependent and that their correction is always complicated when the analyte ILIS is unavailable. The use of the own analyte ILIS is surely the best option for satisfactory correction in all sample types, but this assumption would need to be supported by experimental data as some labelled compounds (e.g. high degree of deuterated isotopes) can have different physico-chemical behavior than the analyte [26]. In multi-residue methods, it uses to be unpractical to correct each compound with its own ILIS due to the high cost and the low commercial availability of ILIS reference standards. In these cases, the use of a few ILIS to compensate matrix effects for all compounds may be an alternative in the environmental field, although the satisfactory correction for all samples analyzed cannot be ensured. The choice of an appropriate internal standard is crucial in terms of compensation for matrix effects [27-29], although it seems insufficient to test it in just one given sample, due to the large variability in environmental waters and wastewaters. Thus, the study of matrix effects should be made in a notable number of samples trying to cover different situations and sample matrix compositions.

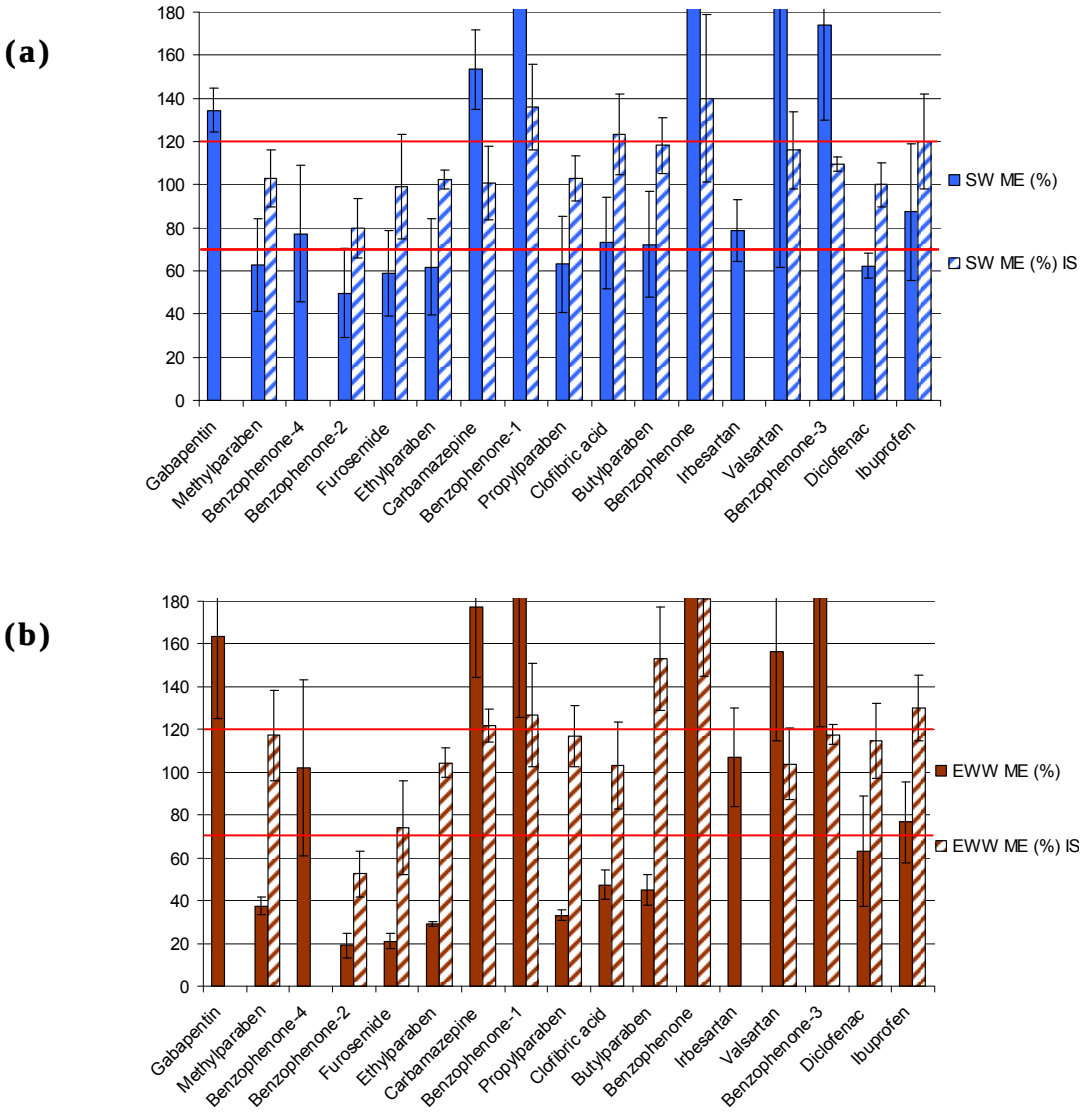


Fig. 3. Average matrix effects (ME) for (a) five different surface water samples and (b) five different effluent wastewater samples, before and after correction with ILIS. *Ethylparaben-d4*: used to correct for ethylparaben, methylparaben, benzophenone-2, furosemide, propylparaben, clofibric acid*, butylparaben. *Benzophenone-3-d5*: used for benzophenone-3, carbamazepine**, benzophenone-1**, benzophenone. *Valsartan-d8*: used for valsartan. *Diclofenac-d4*: used for diclofenac. *Ibuprofen-d3*: used for ibuprofen. * Correction made with diclofenac-d4 in EWW analysis.** Correction made with valsartan-d8 in EWW analysis.

3.5. Method validation

The method linearity was studied in the range 1–100 µg/L (these values corresponded to 0.01–1 µg/L in the water samples, taking into account the 100-fold pre-concentration factor applied along the sample procedure). Calibration curves showed satisfactory correlation coefficients (greater than 0.99) and residuals were lower 30% for all compounds.

For validation purposes, each of the 10 water samples tested (five SW and five EWW samples) was spiked at two concentration levels. Experiments were performed in duplicate. In order to evaluate simultaneously the SPE recovery (RE) and the matrix effect (ME), the overall process efficiency (PE) was determined. Thus, the ratio between the responses obtained for samples spiked before SPE and the response of the standard in solvent was determined [25] in the method validation.

"Blank" samples, spiked only with the ILIS mix, were also processed to subtract the responses of the target compounds that were present in the samples used for validation. PE experimental values, after correction with ILIS, for the five SW samples and the five EWW samples tested are shown inTable 2 and Table 3. ILIS correction was performed on the basis on the results obtained in the previous sections.

The method was tested at two concentration levels (0.05 and 0.5 µg/L) in SW. At the lowest concentration, ibuprofen could not be validated due to the poor sensitivity. A few compounds could not be validated in all the SW samples tested due to the high analyte concentration found in some of the "blank" SW samples used in the validation (e.g. methylparaben, furosemide, propylparaben).

Recoveries in SW (calculated as PE) were satisfactory (between 70% and 120%) for most of the compounds at the two spiking levels. Only benzophenone-4 and irbesartan were quantified without ILIS. Among the remaining compounds, five of them were corrected with their own ILIS, and the rest using an "analogue" ILIS. The use of analogue ILIS was, in general, satisfactory, but could not be always assured appropriate correction in all SW tested. For example, when carbamazepine was corrected with benzophenone-3-d_5, satisfactory recoveries were obtained except for two of the SW samples. Recoveries significantly above 100% were obtained for benzophenone-1 without ILIS correction at both spiking levels (data not shown), which was in agreement with the signal enhancement observed in the matrix effect study. The use of benzophene-3-d_5 as ILIS could not efficiently correct matrix effects, especially at the lowest level.

Table 2
Method validation for surface waters. Process efficiency (%) for five different SW* samples analyzed in duplicate.

Compound	t_R (min)	SW 1		SW 2		SW 3		SW 4		SW 5		Average PE(%)	Average PE(%)	LOQ (ng/L)	ILIS used
		PE (%)	PE (%)	PE (%)	PE (%)	PE (%)	PE (%)	PE (%)	PE (%)	PE (%)	PE (%)				
		0.05 µg/L	0.5 µg/L	0.05 µg/L	0.5 µg/L	0.05 µg/L	0.5 µg/L	0.05 µg/L	0.5 µg/L	0.05 µg/L	0.5 µg/L	0.05 µg/L**	0.5 µg/L**		
Gabapentin	2.01	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	-	-
Methyparaben	3.91	[b]	103	[b]	84	120	114	112	109	71	112	101 (26)	104 (12)	4[c]	Ethylparaben-d_4
Benzophenone-4	4.06	100	106	113	139	[b]	62	71	90	72	66	89 (23)	93 (34)	3	-
Benzophenone-2	4.64	95	71	91	84	65	72	102	89	72	57	85 (19)	75 (17)	4	Ethylparaben-d_4
Furosemide	4.73	68	68	72	76	[b]	73	118	126	92	92	88 (26)	87 (27)	9	Ethylparaben-d_4
Ethylparaben	5.01	99	94	114	102	104	104	109	107	101	101	105 (6)	102 (5)	2[c]	Ethylparaben-d_4
Carbamazepine	5.52	94	88	97	88	125	141	107	103	138	132	112 (17)	110 (22)	0.3	Benzophenone-3-d_5
Benzophenone-1	6.31	307	113	363	124	375	187	343	136	219	89	321 (20)	130 (28)	2	Benzophenone-3-d_5
Propylparaben	5.96	99	91	[b]	97	103	97	121	115	95	104	105 (11)	101 (9)	0.9	Ethylparaben-d_4
Clofibric acid	6.41	105	104	119	119	141	143	133	148	104	127	120 (14)	128 (14)	3	Ethylparaben-d_4
Butylparaben	6.81	108	96	126	132	150	117	149	122	88	105	124 (22)	114 (12)	0.9	Ethylparaben-d_4
Benzophenone	6.72	[b]	33	97	84	[b]	74	106	120	156	127	120 (27)	88 (43)	0.4	Benzophenone-3-d_5
Irbesartan	6.85	67	79	92	79	[b]	59	115	79	69	70	86 (26)	73 (12)	0.2	-
Valsartan	7.08	93	106	98	95	[b]	[b]	107	106	122	126	105 (12)	108 (12)	1	Valsartan-d_8
Benzophenone-3	7.46	97	99	103	102	94	107	117	108	122	122	107 (12)	108 (8)	0.8	Benzophenone-3-d_5
Diclofenac	7.75	86	105	104	97	73	89	116	117	96	101	95 (17)	102 (10)	6	Diclofenac-d_4
Ibuprofen	7.91	[a]	127	[a]	100	[a]	116	[a]	130	[a]	96	[a]	114 (14)	120	Ibuprofen-d_3

a: Not estimated due to the poor sensitivity.

b: Not estimated due to the high analyte levels found in the “blank” sample.

c: LOQ determined from the “blank” sample chromatogram (non-spiked).

* Surface water samples used for validation: Mijares river; Jucar river; Albufera lake; Clot de Burriana lake; Tavernes de la Valldigna lake.

** Average PE for the five SW samples analyzed in duplicate, and relative standard deviation in brackets (n = 10).

Table 3
Method validation for effluent wastewaters. Process efficiency (%) for five different EWW samples analyzed in duplicate.

	EWW 1		EWW 2		EWW 3		EWW 4		EWW 5					
Compound	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**PE** (%)	**Average PE**(%)	**Average PE**(%)	**LOQ**	**ILIS used**
	0.1 µg/L	**0.5** µg/L	**0.1** µg/L	**0.5** µg/L	**0.1** µg/L	**0.5** µg/L	**0.1** µg/L	**0.5** µg/L	**0.1** µg/L	**0.5** µg/L	**0.1** µg/L**	**0.5** µg/L**	**(ng/L)**	
Gabapentin	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	-	-
Methyparaben	79	68	150	128	172	113	104	108	202	111	141 (35)	106 (21)	30	Ethylparaben-d4
Benzophenone-4	81	76	[b]	54	242	109	74	72	[b]	[b]	132 (72)	78 (29)	17[c]	-
Benzophenone-2	60	56	65	54	67	46	72	49	58	54	64 (9)	52 (8)	24	Ethylparaben-d4
Furosemide	79	75	[b]	75	102	61	98	55	[b]	58	93 (13)	65 (15)	45[c]	Ethylparaben-d4
Ethylparaben	103	111	97	104	98	83	104	96	104	105	101 (3)	100 (11)	16	Ethylparaben-d4
Carbamazepine	133	146	122	122	144	146	138	141	124	135	132 (7)	138 (7)	3[c]	Valsartan-d8
Benzophenone-1	184	181	113	108	146	124	123	112	133	111	140 (20)	127 (24)	40	Valsartan-d8
Propylparaben	131	121	129	111	105	88	111	98	131	90	121 (10)	102 (14)	15[c]	Ethylparaben-d4
Clofibric acid	127	134	98	132	127	118	96	104	75	108	105 (21)	119 (11)	26[c]	Diclofenac-d4
Butylparaben	155	160	143	139	134	104	144	119	142	103	144 (5)	125 (20)	14	Ethylparaben-d4
Benzophenone	304	161	[b]	[b]	117	135	[b]	113	151	153	191 (52)	141 (15)	33[c]	Benzophenone-3-d5
Irbesartan	60	82	[b]	61	165	85	56	63	[b]	52	94 (66)	69 (21)	2[c]	-
Valsartan	65	114	70	106	119	119	119	120	72	105	89 (31)	113 (6)	4[c]	Valsartan-d8
Benzophenone-3	115	113	113	113	120	106	101	102	120	110	114 (8)	109 (4)	7	Benzophenone-3-d5
Diclofenac	[b]	123	[b]	93	[b]	124	[b]	107	[b]	[b]	[b]	112 (13)	95[c]	Diclofenac-d4
Ibuprofen	[a]	134	[a]	122	[a]	111	[a]	129	[a]	130	[a]	125 (7)	280	Ibuprofen-d3

a: Not estimated due to the poor sensitivity.

b: Not estimated due to the high analyte levels found in the “blank” sample.

c: LOQ determined from the “blank” sample chromatogram (non-spiked)

** Average PE for the five EWW samples analyzed in duplicate, and relative standard deviation in brackets (n = 10).

It is important to remark that, in a few cases, recoveries varied significantly from one sample to another (e.g. for furosemide, individual recoveries were in the range 68–126% at the highest level assayed). This situation was only observed when an analogue ILIS was used because the composition of aqueous samples is never the same, and unexpected matrix effects might occur.

Regarding EWW, the method was also tested in five different samples at two fortification levels each (0.1 and 0.5 µg/L). All analytes were quantified using ILIS with the exception of benzophenone-4 and irbesartan, which presented acceptable recoveries without correction (see Table 3). A few compounds could not be validated in all the five samples, due to the high concentrations found in the "blank", especially in one of the samples (named as EWW 2). Recoveries were mostly satisfactory at the two levels assayed. A few compounds showed values higher than 120% in some of the EWW tested when the own analyte ILIS was unavailable (e.g. methylparaben, carbamazepine, benzophenone-1, butylparaben), illustrating that matrix effects for a sample type (e.g. effluent wastewater) do not remain constant along time and for all the samples analyzed, and can suffer notable variations. Therefore, a satisfactory correction could not be ensured for all the samples analyzed when using analogues ILIS, even though the method was tested in a notable number of water samples.

A particular case was benzophenone, as this compound presented poor extraction efficiency (RE value around 50%) and remarkable signal enhancement (ME around 250%), leading to a PE value near 120%. Thus, the term "process efficiency" (PE) in this particular analyte might give a biased overall view of the method reliability.

Instrumental LODs are shown in Table 1. They ranged from 0.3 to 10.6 pg, with the exception of ibuprofen. For this compound LOD was significantly higher than for the rest of analytes. The reason was its poor fragmentation, and that only a low sensitive transition could be monitored, hampering its confirmation.

Concerning LOQs, they ranged from 0.2 to 9 ng/L for SW, and were around 10-fold higher than for the rest of analytes. The reason was its poor fragmentation, and that only a low sensitive transition could be monitored, hampering its confirmation.

3.6. Application to environmental and waste water samples

The method developed in this paper was applied to investigate the presence of 17 PPCPs in different types of water: reservoir water (Colombia), and SW and EWW (Spain).

In every sequence of analysis, the calibration curve was injected twice, at the beginning and the end of the sample batch. Moreover, quality control samples (QCs) were included in every sample sequence. QCs consisted on SW, EWW or reservoir water spiked at 0.5 μg/L. They were prepared randomly selecting one of the water samples analyzed within the batch, and were analyzed following the same analytical procedure than samples. QC recoveries in the range 60–120% were considered as satisfactory (Tables 4 and Table 5).

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q_1 and q_2) transitions. The finding was considered as true positive when the experimental ion-ratio was within the tolerance range [30], and the retention time in the sample within ± 2.5% the retention time, when compared with a reference standard.

A great variety of PPCPs were detected in a wide range of concentrations in samples from Spain (Table 4). In SW, 14 out of 17 PPCPs were detected at least once. Among them, irbesartan, benzophenone, benzophenone-3, methylparaben and propylparaben were quantified in all SW samples. On the contrary, clofibric acid, benzophenone-2 and butylparaben were not detected in any of the samples. The highest concentrations found were for valsartan (up to 6.3 μg/L). These results are in fairly good agreement with data reported by other authors. For instance, in a study carried out by Kasprzyk-Hordern et al. [31], the pharmaceuticals ibuprofen, diclofenac, gabapentin, furosemide and valsartan were also frequently found in SW samples from United Kingdom and their concentration levels were similar to those found in our study. Regarding PCPs, in other works highest concentrations were found for methylparaben and benzophenone-4 [10,31], which is in accordance with the present study. The presence of pharmaceuticals in “Albufera de Valencia” lake has been previously reported in literature [32], concretely carbamazepine (2.2–31 ng/L), ibuprofen (n.d.−3913 ng/L), clofibric acid (n.d.−71.4 ng/L) and diclofenac (n.d. −260.9 ng/L), among other compounds. These data are in the line of the present work, where carbamazepine (12 ng/L) and ibuprofen (<LOQ) were also found in the same area. The most polluted source was Jucar river (in Valencia) where 13 out of 17 targeted PPCPs were detected, the majority of them at the highest concentration levels found in this work.

Table 4
Summary of the results obtained for target PPCPs in SW and EWW (Spain).

Target compounds	SW (n = 11)					EWW (n = 11)				
	Minimum level (ng/L)	Maximum level (ng/L)	Median concentration (ng/L)	Positive samples (%)	QC (%)	Minimum level (ng/L)	Maximum level (ng/L)	Median concentration (ng/L)	Positive samples (%)	QC (%)
Gabapentin	[a]	[a]	[a]	[a]	-	[a]	[a]	[a]	[a]	-
Ibuprofen	<LOQ	939	135	45	88	633	1450	1042	18	85
Diclofenac	20	330	82	55	105	185	1153	721	100	100
Clofibric acid	-	-	-	0	102	101	479	120	45	117
Valsartan	24	6260	163	82	101	73	5899	585	100	118
Irbesartan	8	651	57	100	95	462	1309	889	100	99
Carbamazepine	3	176	13	72	107	100	305	153	100	145c
Furosemide	<LOQ	175	28	64	72	72	1036	212	100	79
Benzophenone	115	787	195	100	63	476	1068	784	100	b
Benzophenone-1	221	221	221	10	120	-	-	-	0	185c
Benzophenone-2	-	-	-	0	72	-	-	-	0	71
Benzophenone-3	5	107	7	100	93	8	167	23	100	106
Benzophenone-4	14	952	68	82	96	141	2032	1000	100	93
Methylparaben	6	208	30	100	94	<LOQ	66	<LOQ	100	108
Ethylparaben	2	4	3	18	93	-	-	-	0	97
Butylparaben	-	-	-	0	99	-	-	-	0	113
Propylparaben	2	81	15	100	88	<LOQ	21	<LOQ	100	94

a: For gabapentin, due to its low recovery, only its presence could be reported.
b: Not estimated due to the high analyte levels found in the sample used for preparing the QC.
c: Value out of the tolerance range established (60 – 120%).

Regarding EWW, 10 of the target PPCPs were detected in all the samples (benzophenone, benzophenone-3, benzophenone-4, methylparaben, propylparaben valsartan, irbesartan, diclofenac, carbamazepine and furosemide). On the contrary, four compounds were not detected in any EWW sample: benzophenone-1, benzophenone-2, ethylparaben and butylparaben, which was in general agreement with data obtained in SW (benzophenone-1 and ethylparaben were found in only one and two surface waters, respectively). The highest levels were found for benzophenone-4 and valsartan, which were in the range 0.14–2 and 0.07–5.9 μg/L, respectively. Valsartan was by far the compound present at higher levels in both SW and EWW samples. As expected, concentrations in EWW were usually higher than in SW. In agreement with other studies [10] and [31], preservatives were found at very low levels. This was also observed for benzophenone-1, benzophene-2 and benzophenone-3, whilst benzophenone-4 was one of the compounds found at higher concentrations.

For gabapentin, due to its low recovery, only qualitative data were obtained. This compound was detected in four SW and in all EWW samples analyzed. It was confirmed by the presence of chromatographic peak at the three SRM transitions acquired for this compound.

In relation to reservoir water from Colombia, both water sources showed less contamination by PPCPs than surface water from Spain. In fact, there were no evidences on the presence of gabapentin, ibuprofen, diclofenac, clofibric acid, valsartan, irbesartan, carbamazepine, furosemide and benzophenone-2 in any reservoir. A summary of the results obtained is shown in Table 5. On the contrary, the occurrence of parabens and two benzophenones was highly relevant in both reservoirs. In reservoir 1, methylparaben and propylparaben were present in all samples in a wide range of concentrations. These compounds were also found in most of the samples of the reservoir 2 (93% of positive samples). This is in accordance with previous studies where methylparaben was the preservative most frequently detected in river waters from the United Kingdom [31]. Ethylparaben was found in around 70% of samples from reservoir 1 and in all samples from reservoir 2, although at very low levels, commonly below 10 ng/L. Benzophenone and benzophenone-3 were detected in both reservoirs in a notable number of samples. On the contrary, benzophenone-1 and benzophenone-2 were only detected in the reservoir 1 in just one sample.

Table 5
Summary of the results obtained for target PPCPs in reservoir water (Colombia).

Target compounds	Reservoir 1 (n = 22)					Reservoir 2 (n = 29)				
	Minimum level (ng/L)	Maximum level (ng/L)	Median concentration (ng/L)	Positive samples (%)	QC (%)	Minimum level (ng/L)	Maximum level (ng/L)	Median concentration (ng/L)	Positive samples (%)	QC (%)
Gabapentin	-	-	-	0	-	-	-	-	0	-
Ibuprofen	-	-	-	0	118	-	-	-	0	120
Diclofenac	-	-	-	0	110	-	-	-	0	104
Clofibric acid	-	-	-	0	122	-	-	-	0	117
Valsartan	-	-	-	0	111	-	-	-	0	105
Irbesartan	-	-	-	0	110	-	-	-	0	107
Carbamazepine	-	-	-	0	100	-	-	-	0	105
Furosemide	-	-	-	0	116	-	-	-	0	120
Benzophenone	2	4	3	60	138[c]	4	26	10	82	149[c]
Benzophenone-1	6	6	6	5	111	-	-	-	0	115
Benzophenone-2	-	-	-	0	100	-	-	-	0	112
Benzophenone-3	2	184	4	95	101	3	225	6	58	103
Benzophenone-4	5	5	5	5	99	-	-	-	0	105
Methylparaben	27	537	88	100	130[c]	17	204	49	93	129[c]
Ethylparaben	4	41	9	68	110	3	7	4	100	110
Butylparaben	13	55	25	7	109	-	-	-	0	99
Propylparaben	13	57	25	100	109	11	160	29	93	107

c: Value out of the tolerance range established (60 – 120%).

Recoveries for the Quality Control samples (QCs) included in every sequence of analysis were, in general, satisfactory, within a range of 60–120% (see Table 4 and Table 5). Only a few exceptions were observed in EWW and in the reservoir water samples. In all these cases, QCs recoveries were higher than 120% and corresponded to analytes that were corrected with an analogue ILIS. A correction factor was applied when the compound was detected and quantified in the samples (five out of six cases).

As an illustrative example, Fig. 4 shows a positive finding of methylparaben, propylparaben, benzophenone and benzophenone-3 in one of the water samples collected at reservoir 2. From the data obtained on PCPs in the water from reservoirs, we did not observe specific trends in the concentrations of these compounds at the different depths considered in the water column.

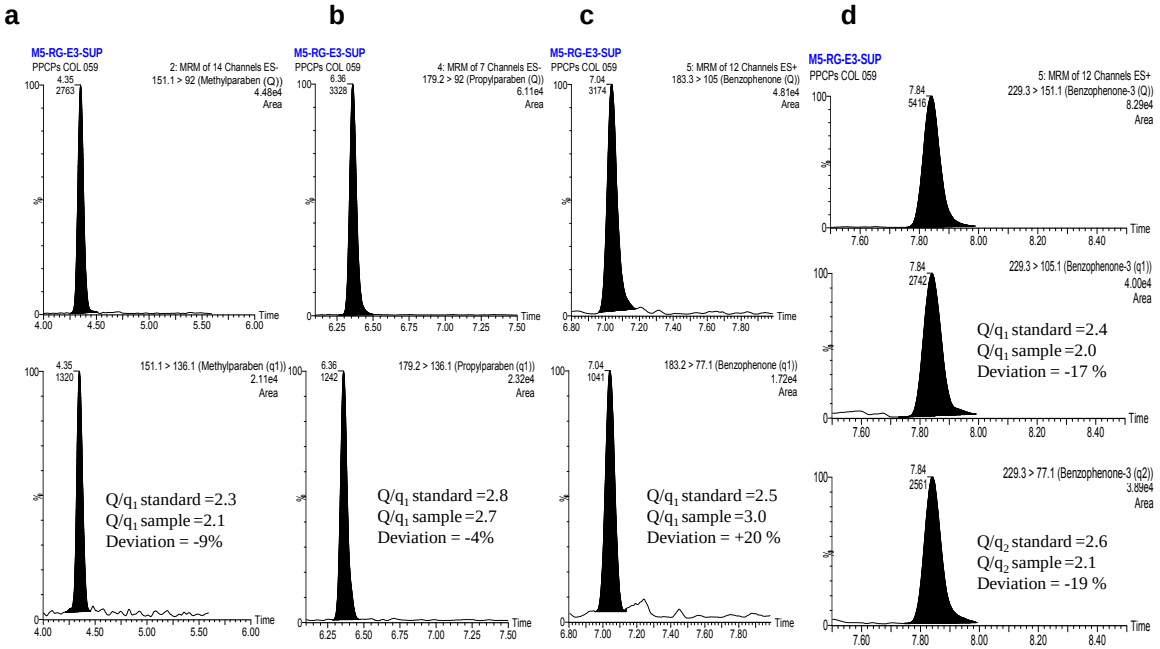


Fig. 4. UHPLC-MS/MS chromatograms for selected analytes for a reservoir water sample. Methylparaben (141 ng/L), propylparaben (142 ng/L), benzophenone (7 μg/L), benzophenone-3 (6 ng/L).

None of the target pharmaceuticals was detected in the reservoir waters from Colombia. This was unexpected, especially for diclofenac and ibuprofen, which seem to be widely consumed in this country. The reason might be found in the high dilution taking place due to the large capacity of these reservoirs.

The influence of human activities in areas near the Colombian reservoirs seems clear in the case of PCPs (for example, reservoir 1 is used as a recreation place and it is surrounded by residences), but more research and data would be required to understand the fate and behavior of pharmaceuticals on this aquatic environment.

The method developed will be implemented in the laboratory of GDCON, University of Antioquia, from 2012. Thus, it is expected that more data will be available on PPCPs in the aquatic environment of this area in the near future.

4. Conclusions

In this paper a multi-residue method for the simultaneous quantification and confirmation of 17 emerging contaminants, including widely used pharmaceuticals and personal care products, has been developed and applied to surface water samples and effluent wastewater from Spain and Colombia.

The method has been evaluated and validated in a total of 10 water samples (five surface waters and five effluent wastewaters). This number of samples, higher than usual in most method validations reported for PPCPs, was chosen in order to have a wider overview of the method performance in different water samples. The matrix effects study performed in this work shows severe signal suppression/enhancement, especially in some of the EWW tested. The correction with analyte ILIS was satisfactory in all sample types. The use of analogues ILIS was rather satisfactory, although it could not ensure appropriate correction in all the analyte/water samples combinations, as matrix effects are both compound and matrix dependent. As well known, the composition of water samples is never the same and notable changes can occur along the time and as a function of their origin. As a consequence, matrix effects that commonly affect LC–MS/MS methods can be rather different from one sample to other. Thus, the selection of appropriate analogue ILIS, when the analyte ILIS is not available, is

controversial and should be only made after a careful study on matrix effects in a considerable number of water samples of different types and origin.

The results obtained in this work after the application of the method to 73 water samples (surface: rivers, lakes and reservoirs, and effluent wastewater) showed the wide majority of the target analytes were present in the samples analyzed. The importance of including personal care products, which have received less attention than pharmaceuticals during the last years, is evidenced by the widespread occurrence of these compounds in the samples under study. To the best of our knowledge, the presence and distribution of PPCPs in Colombia have not been reported, and this is the first publication providing data on PPCPs in this country.

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2.5.3 Discusión de los resultados (artículo científico 4)

Optimización de las condiciones MS/MS

Cinco de los compuestos estudiados (valsartan, irbesartan, benzofenona-1, benzofenona-2 y benzofenona-3) se ionizaron en modo positivo y negativo. De ellos tan sólo se escogió el modo de ionización negativo para la benzofenona-2 porque la sensibilidad en este modo de trabajo era mayor. A modo de ejemplo, en la Figura 2.12 se muestran los espectros de la benzofena-2 y del irbesartan en ambos modos de ionización.

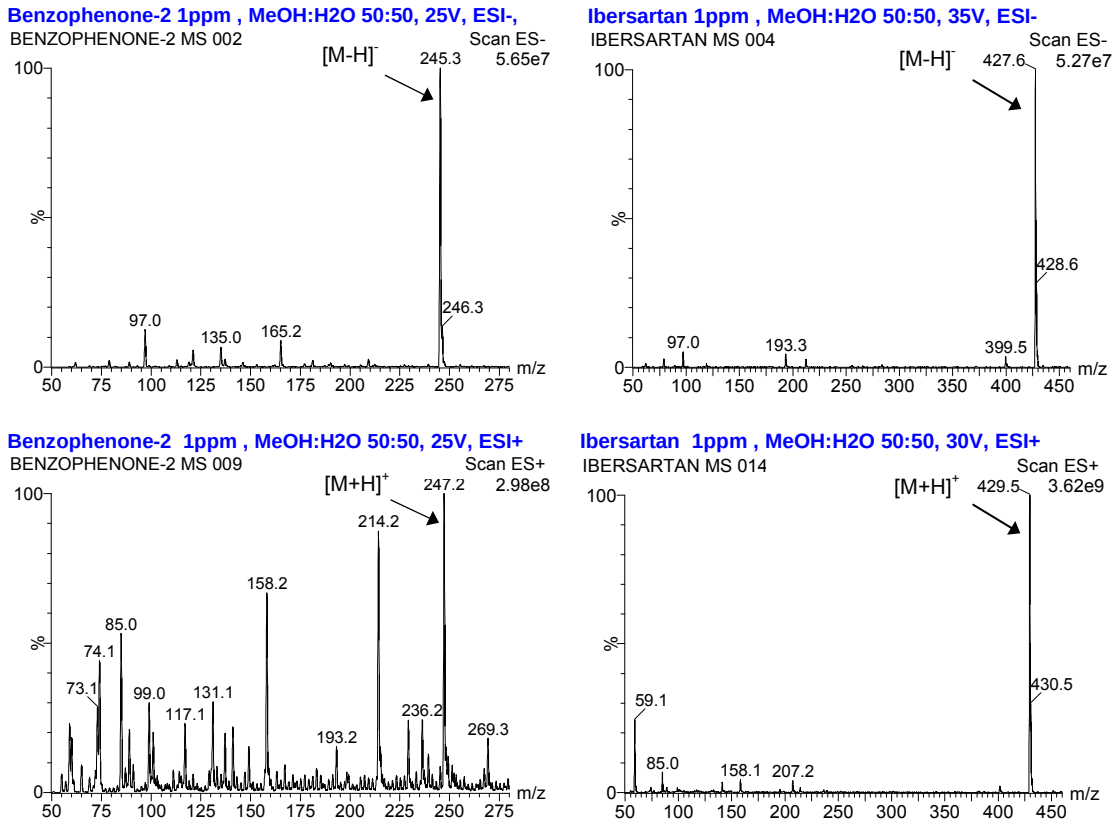

Figura 2.12 Espectros MS en modo de ionización negativo (arriba) y positivo (abajo) obtenidos al infusionar un patrón de (a) benzofenona-2 y (b) irbesartan.

Finalmente, diez compuestos se ionizaron en modo negativo y se seleccionó como ion precursor $[M-H]^-$. Para los siete restantes, determinados en modo positivo, el ion más abundante fue $[M+H]^+$. El espectro de infusión *full scan* del diclofenaco y del ácido clofíbrico mostró dos iones predominantes, correspondientes a los iones $[M-H]^-$ con ^{35}Cl y ^{37}Cl. Así, para estos compuestos se trabajó con dos iones precursores distintos.

En todos los casos en los que fue posible se seleccionaron tres transiciones por compuesto con el fin de asegurar de manera fiable su identidad.

Optimización del proceso de extracción

Se compararon dos cartuchos con diferente mecanismo de retención: Oasis HLB y Oasis MCX. Se comprobó la eficacia de retención de ambos sorbentes para los analitos seleccionados en agua superficial y efluente urbano.

Utilizando el cartucho HLB se obtuvieron buenas recuperaciones para todos los compuestos excepto para gabapentina, benzofenona y benzofenona-3. El cartucho MCX resultó ser adecuado para la retención de gabapentina pero no para los otros dos compuestos. Esto se debe a las diferentes características físico-químicas de los analitos. La gabapentina es una molécula pequeña y polar que posee grupos ácidos y básicos. Cuando se acidifica el pH de la muestra de agua, los grupos básicos se protonan y quedan retenidos en los cartuchos MCX.

En general, las recuperaciones utilizando el cartucho HLB fueron más elevadas tanto en el agua superficial como en efluente y por ello se escogió este cartucho.

Optimización del método. Estudio del efecto matriz

En todos los trabajos presentados en esta Tesis, pero especialmente en este, se puso especial interés en la evaluación del efecto matriz. Como es bien sabido, la presencia de interferentes puede provocar importantes errores en la cuantificación de

los analitos, que ponen en riesgo la calidad de los resultados. Por ese motivo, se debe conocer en qué medida la presencia de dichos interferentes, presentes en la muestra, afecta a la ionización de los analitos para tratar de corregir sus efectos o, al menos, minimizarlos. Cabe recordar que el efecto matriz depende de la combinación analito/matriz y, por tanto, debería evaluarse en todos los tipos de muestra que se deseen estudiar (ej. agua superficial, agua de efluente y agua de influente).

El estudio del efecto matriz se realizó en cinco aguas superficiales y en cinco efluentes. Para ello, se compararon las respuestas obtenidas con un patrón preparado en agua HPLC con las respuestas de las muestras fortificadas al mismo nivel (50 μg/L, equivalente a 0.5 μg/L en muestra) tras el proceso de extracción por SPE.

Para la mayoría se observó una supresión de la señal, que fue más acusada en el caso de las muestras de efluente. Para tratar de corregir la supresión/exaltación de la señal de todos ellos se decidió evualuar la eficacia de corrección de cinco patrones internos marcados isotópicamente. Recordemos que hoy en día éste es el método de corrección más utilizado en LC-MS/MS. Sin embargo, como ya se ha señalado anteriormente, su aplicación en el caso de métodos multirresiduales conlleva una cierta dificultad pues resulta poco probable poder corregir cada analito con su propio compuesto marcado.

Como era de prever, los efectos matriz para los cinco compuestos cuya molécula marcada se utilizó como patrón interno se corrigieron satisfactoriamente en todas las aguas analizadas.

En aquellos compuestos para los que no se disponía de su propio analito marcado, el criterio de selección se basó en la similitud entre la estructura química del analito y el compuesto marcado. Así, por ejemplo, el etilparabeno-d_4 se usó para la corrección del grupo de los parabenos. En ausencia de un analito marcado similar, la elección se basó en el tiempo de retención más próximo al del analito. Un ejemplo fue el uso del etilparabeno-d_4 para corregir a la furosemida.

Los analitos no están siempre afectados por el mismo grado de supresión o de exaltación y su comportamiento varía en función de la matriz, e incluso en un mismo tipo de agua puede existir variabilidad. Un ejemplo se halló en la corrección de la carbamazepina. En agua superficial, al utilizar la benzofenona-3-d_5 como patrón interno análogo, se corrigió la exaltación de la señal de este compuesto. En cambio, su uso no resultó adecuado en la mayoría de las muestras de efluente y sí lo fue el valsartán-d_8.

Los resultados obtenidos en este estudio confirman nuevamente que el uso de análogos estructurales como patrones internos no siempre asegura la corrección del efecto matriz. Por ese motivo, la elección de un análogo sólo debe realizarse tras un estudio del efecto matriz realizado en un número considerable de muestras de distinto tipo y origen.

Validación del método

El método fue validado en cinco aguas superficiales a dos niveles de fortificación (0.05 y 0.5 µg/L) y en cinco efluentes, también a dos niveles (0.1 y 0.5 µg/L). Cada muestra se fortificó antes de la etapa de SPE y su respuesta se comparó con la de un patrón en solvente. La recuperación obtenida de este modo tiene en cuenta la eficacia del proceso de extracción (si se han producido pérdidas o no) y el efecto matriz (exaltación/supresión de la señal).

En las *Tablas 2 y 3, artículo científico 4* se muestran los valores de recuperación obtenidos en las diez aguas utilizadas, a los dos niveles de fortificación. Para dar una visión global del proceso, en las tablas se ha indicado también el valor promedio de recuperación para cada matriz a cada nivel junto con el valor de RSD. Como era de esperar, los resultados de los compuestos que se corrigieron con su molécula marcada fueron satisfactorios. El uso de un marcado análogo mejoró las recuperaciones excepto en algunos casos concretos (véanse las *Tablas 2 y 3, artículo científico 4*). Este tipo de situaciones problemáticas pueden producirse con mayor frecuencia de la esperada cuando se analizan muestras de agua debido a la variabilidad en su composición. Por

ello, para comprobar que el uso de marcados análogos resulta fiable se deberían incluir varias muestras de control de calidad (QCs) en cada secuencia de análisis.

Análisis de muestras. Resultados en aguas superficiales y en efluentes

La metodología descrita se aplicó a muestras de agua superficial recogidas en ríos, lagos y embalses y a muestras de efluente urbano. Las aguas de embalses procedían de Colombia, en concreto de dos situados en el departamento de Antioquia que actualmente se utilizan para el abastecimiento de agua potable de la zona. La presencia de contaminantes en las aguas es siempre un motivo de preocupación pero lo es todavía más cuando el agua se utiliza para consumo humano. El número de contaminantes encontrados en las muestras de los embalses fue menor que en el resto de muestras analizadas. En estas aguas tan sólo se detectaron productos de cuidado personal, destacando la presencia de parabenos y en concreto, la de metilparabeno. Este compuesto se detectó en 49 de las 51 muestras en un amplio rango de concentraciones.

El resto de las aguas superficiales se tomaron en distintos puntos de la Comunidad Valenciana. Se detectaron todos los compuestos excepto tres de ellos (ácido clofíbrico, benzofenona-2 y butilparabeno). En cambio sí se hallaban, entre otros, el irbesartan y valsartán. Se trata de compuestos antihipertensivos que no se suelen analizar en las aguas medioambientales pero en este estudio se detectaron casi todas las muestras (100% y 82% respectivamente). Además, la concentración del valsartán fue muy superior a la del resto de PPCPs, siendo su concentración máxima hallada 6.3 μg/L.

La presencia de los productos de cuidado personal en las aguas de la Comunidad Valenciana también resultó destacable. Cuatro de ellos (benzofenona, benzofenona-3, metilparabeno y propilparabeno) se identificaron en todas las muestras analizadas. Los niveles de concentración de las benzofenonas fueron más elevados en general.

En las aguas de efluente, más de la mitad de los compuestos se detectaron en todas las muestras, siendo sus concentraciones superiores a las de las aguas superficiales. Excepto para benzofenona y benzofenona-4, cuya concentración media fue en torno a 800 y 1000 ng/L respectivamente, los niveles obtenidos para los productos de cuidado personal resultaron más bien bajos.

Entre los fármacos, los mayores valores de concentraciones, expresadas como medianas, correspondieron a irbesartán e ibuprofeno (900 y 1000 ng/L aproximadamente). Sin embargo, el ibuprofeno se detectó sólo en el 18% de las muestras analizadas mientras que el irbesartán se encontró en la totalidad de las muestras de efluente.

2.5.4 Bibliografía

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Capítulo 3

Investigación de metabolitos y de productos de transformación de fármacos en aguas

3.1. Introducción

Como se ha visto en el Capítulo 2, la presencia de los fármacos en el medio ambiente es un tema de gran interés. En los últimos años se han realizado numerosos estudios para determinar la presencia y el destino de estos contaminantes en el agua. En cambio, la presencia de los metabolitos y de los productos de transformación ha quedado relegada a un segundo plano.

Los metabolitos son los productos que se forman cuando los fármacos experimentan una transformación en el organismo humano o animal en el proceso de metabolización. Tanto fármacos como metabolitos pueden sufrir transformaciones biológicas y/o físico-químicas durante su transporte a las EDAR a través de la red de alcantarillado, durante el tratamiento de depuración y en el medio ambiente. Los compuestos que se forman mediante la influencia de cualquiera de estos agentes reciben el nombre de productos de transformación. Generalmente, cuando las transformaciones se producen en el medio ambiente se habla de degradación (Jjemba, 2006), aunque no siempre conduzcan a la completa degradación del producto a especies inorgánicas (ej. agua, CO_2, HCl, etc.).

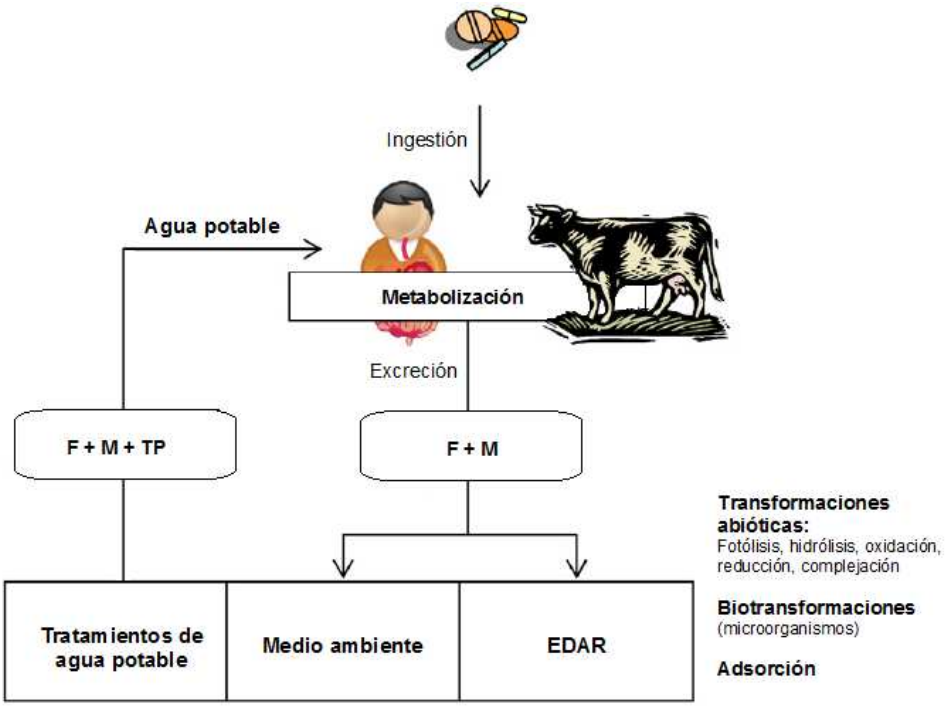

Figura 3.1 Vías de transformación de los fármacos (F: fármaco de partida; M: metabolito; TP: producto de transformación). Modificado de Mompelat, 2009.

El proceso de metabolización se produce en dos fases. En la primera de ellas el compuesto se transforma en una forma más polar y soluble mediante reacciones de oxidación, reducción o hidrólisis que introducen grupos funcionales como -OH, -SH, -NH_2 o –COOH. La segunda fase consiste en reacciones de conjugación en las que un grupo polar o una molécula se une a los compuestos de partida o a los metabolitos formados previamente en la primera fase. La reacción más común es la de glucuronidación en la que el ácido glucurónico se une a los grupos reactivos del compuesto, tales como los grupos fenol, carboxilo, tioles o amina (Pérez, 2007; Mompelat, 2009; Celiz, 2009). Aunque es menos frecuente, también puede unirse un grupo sulfato o un acetilo.

La adición de estos sustituyentes disminuye la liposolubilidad del fármaco y favorece por tanto su eliminación a través de la orina o de las heces. La mayoría de las reacciones de conjugación del organismo tienen lugar en el hígado.

El grado de metabolismo de un fármaco en el cuerpo humano varía de un compuesto a otro. Algunos autores han establecido una clasificación que abarca desde los compuestos que no sufren metabolización o es mínima, hasta aquellos que sufren una transformación casi completa (Jjemba, 2006). En el primer grupo se encuentran, por ejemplo, la gabapentina o el valsartán, y en el último, la carbamazepina y el diazepam.

Mención especial merecen los fármacos conocidos como profármacos. Este término fue propuesto por Albert para referirse a los compuestos inactivos que una vez ingeridos se convierten en una forma activa mediante una reacción metabólica (Albert, 1958). Dicho de otro modo, el compuesto que se ingiere no es el que actúa en el organismo sino la forma activa en la que se transforma. Por ese motivo carece de sentido analizar la presencia de los profármacos en el medio ambiente. En su lugar se analizan los metabolitos activos, por ejemplo, el ácido clofíbrico, el ácido fenofíbrico o el ácido salicílico, en lugar de clofibrato, fenofibrato y ácido acetilsalicílico respectivamente. Estos compuestos han sido hasta hace poco tiempo prácticamente los únicos metabolitos que se incluían en los métodos de análisis.

En general, la información disponible acerca de la presencia de metabolitos en el medio ambiente es escasa. Prueba de ello es que tan sólo unos 30 subproductos (metabolitos y productos de transformación) se han estudiado en los trabajos publicados (Mompelat, 2009). La importancia de su presencia en el medio ambiente todavía no está clara, pero para proteger la calidad del agua, la Agencia Europea de Medicamentos (EMEA) y la Agencia de Alimentos y Medicamentos (FDA) de Estados Unidos establecen que antes de comercializar un nuevo fármaco se debe valorar su riesgo ambiental. Eso implica, entre otros aspectos, conocer el metabolismo del producto así como los porcentajes de excreción del compuesto y de los metabolitos formados.

A día de hoy tampoco existe mucha información sobre la peligrosidad individual y colectiva de los metabolitos y de sus fármacos precursores ni sobre su persistencia en el medio ambiente. Algunos metabolitos pueden ser peligrosos, incluso más que el compuesto de partida (Kostopoulou, 2008), por lo que deben ser incluidos en las metodologías analíticas.

En el caso de los productos de cuidado personal (PCPs) existe la misma problemática ya que en algunos casos los metabolitos y TPs son más persistentes en el medio ambiente que sus precursores (Pedrouzo, 2011). El metabolismo de los PCPs depende de la vía de exposición, bien sea por inhalación, contacto dérmico o ingestión (Jiménez-Díaz, 2011). En algunos PCPs, como los parabenos, su metabolismo a través de la piel humana parece ser menor que en otras especies y hasta puede variar entre las personas (Jewell, 2007; Jiménez-Díaz, 2011).

Los recientes avances en las técnicas analíticas han facilitado la identificación de los fármacos en muestras medioambientales. Estos progresos han permitiendo alcanzar límites de detección lo suficientemente bajos para el análisis de estos contaminantes, especialmente en el caso de los metabolitos que suelen encontrarse a menores concentraciones que los fármacos.

En la mayoría de los estudios publicados se han aplicado métodos analíticos basados en acoplamientos cromatografía-MS. Los analizadores más utilizados (Q y QqQ) requieren la selección previa de los iones que deben medirse (SIM y SRM), por lo que los analitos *target* deben ser seleccionados previamente al desarrollo y validación del método. Sin embargo, el potencial que ofrecen las técnicas de espectrometría de masas de alta resolución para el *screening* y la identificación de metabolitos se ha utilizado en un número muy limitado de trabajos. Por ejemplo, recientemente se ha publicado un artículo basado en el uso de QTOF para el análisis de 100 fármacos, siendo 16 de ellos metabolitos. La lista de compuestos incluye el tiempo de retención, la fórmula molecular y la masa exacta de las moléculas protonadas o desprotonadas y la de sus principales iones (Ferrer, 2012). Esta información resulta de gran utilidad para otros investigadores que trabajen en este campo de análisis.

En esta Tesis, el interés por estudiar los metabolitos surgió a raíz de que algunos de los compuestos investigados en los artículos científicos presentados en el Capítulo 2 no se detectaron en ninguna de las muestras analizadas. Esta situación nos llamó especialmente la atención en el caso de aquellos compuestos que pertenecían a la lista de los fármacos más consumidos en España. Su ausencia podría deberse a la metabolización del fármaco tras ser ingerido o a su transformación en el agua. Por tanto, para poder conocer el impacto de estos compuestos en el medio ambiente se deberían estudiar sus metabolitos y productos de transformación.

A continuación, se presentan tres artículos científicos en los que se ha investigado la presencia de metabolitos y TPs de fármacos en aguas. En los dos primeros (apartado 3.2) el estudio se realizó desde un enfoque cualitativo, haciendo uso de un analizador híbrido cuadrupolo-tiempo de vuelo. En cambio, en el último el enfoque fue cuantitativo usando analizador de triple cuadrupolo (apartado 3.3).

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3.2 Investigación de metabolitos/TPs de fármacos en aguas residuales urbanas mediante cromatografía líquida acoplada a analizador híbrido cuadrupolo-tiempo de vuelo (QTOF)

3.2.1 Introducción

La alta sensibilidad en modo de barrido de iones (modo *scan)* del espectrómetro de masas de tiempo de vuelo (TOF) junto con su elevado poder de resolución y velocidad de barrido, y sobre todo, su capacidad para realizar medidas de masa exacta de los iones detectados, convierten a estos instrumentos en una poderosa técnica analítica para el *screening* e identificación de los compuestos detectados en las muestras.

La resolución es la capacidad de un instrumento para resolver picos muy próximos. En espectrometría de masas este parámetro hace referencia a la capacidad del espectrómetro para distinguir en el espectro de masas las señales correspondientes a iones con una relación m/z similar. Esta capacidad se expresa como poder de resolución y se define como $m/\Delta m$, siendo m la masa nominal de una molécula y Δm la diferencia entre las masas exactas de las moléculas (Marshall, 2008; Hernández, 2012). El poder de resolución se puede definir también para un solo pico, siendo en este caso Δm la anchura del pico a mitad de su altura.

El poder de resolución proporciona información sobre la anchura relativa de los picos y también sobre el rendimiento del analizador, ya que es una medida de la precisión en un amplio rango de m/z (Marshall, 2008). Cuando se indica el poder de resolución de un analizador de masa se debe especificar el valor de masa (m/z) al que se ha estimado y el criterio utilizado para medir Δm. Generalmente, la resolución se suele expresar como FWHM (*full width at half maximum*), es decir, se determina como la anchura del pico a la mitad de su altura, aunque en ocasiones el criterio que se utiliza es el del 10% de valle.

Una elevada resolución permite obtener medidas robustas de masa exacta y, debido a ello, distinguir entre compuestos cuya masa es muy similar. Este es el caso, por ejemplo, de los compuestos isobáricos, que tienen la misma masa nominal pero distinta composición elemental y por tanto, diferente masa exacta. La elevada resolución de los TOF más modernos permite seleccionar ventanas de masa muy

estrechas (por ejemplo, 0.01 - 0.02 Da), facilitando la detección de los compuestos a través del cromatograma generado a partir de la extracción de un ion con una masa específica (XIC). Una ventana más estrecha conduce a una reducción de las interferencias isobáricas y por tanto, la selectividad aumenta.

Por el contrario, en el caso de trabajar con isómeros, debido a que poseen la misma composición elemental y por tanto la misma masa exacta, tan sólo es posible distinguirlos en función de su tiempo de retención y de su fragmentación. En estas situaciones la cromatografía juega un papel muy importante.

El analizador TOF ofrece la posibilidad de investigar la presencia de un compuesto tras haber realizado el análisis y adquirido los datos, es decir, realizar un análisis *a posteriori*. Haciendo uso de la terminología propuesta por nuestro grupo, a esta metodología de trabajo se le denomina análisis *post-target* y resulta adecuada para el *screening* y/o identificación de un amplio número de contaminantes, la confirmación de posibles muestras positivas analizadas mediante otras técnicas y la elucidación de compuestos desconocidos (Hernández, 2012). Todo esto es posible porque el espectro de masas que se adquiere en un análisis contiene información de toda la muestra y con medidas de masa exacta (*accurate-mass full-spectrum acquisition*).

Una de las ventajas del modo *post-target* es la posibilidad de realizar un análisis retrospectivo. Este permite detectar e identificar otros compuestos de interés, que en un principio no se habían considerado, en muestras previamente analizadas, revisando los cromatogramas y espectros de masa adquiridos. Para ello, los iones con una m/z específica se extraen del cromatograma TIC (*Total Ion Current*), generando los cromatogramas XIC donde la presencia del compuesto en la muestra genera el correspondiente pico cromatográfico. Además, el análisis puede realizarse sin utilizar un patrón de referencia llegando a identificaciones tentativas muy fiables. Así, este modo de trabajo permitiría, en un principio, analizar un número ilimitado de contaminantes de interés incluso meses o años después de haber realizado el análisis (Hernández, 2011) siempre y cuando las condiciones de análisis escogidas (tratamiento

de la muestra, cromatografía e ionización) resulten adecuadas para los nuevos contaminantes.

Los analizadores TOF miden generalmente la masa exacta de las moléculas (des)protonadas mientras que los analizadores híbridos, como el QTOF, proporcionan además información relevante sobre iones fragmento del compuesto. En el QTOF, la presencia de un filtro cuadrupolar previo permite realizar experiencias en modo MS/MS. Además, también se puede trabajar en el modo de análisis llamado MS^E en el que se adquieren simultáneamente dos funciones, una a baja energía de colisión (*Low Energy*, LE) y otra a alta energía (*High Energy*, HE). En el primer caso la fragmentación es mínima mientras que a alta energía de colisión las moléculas se fragmentan en la celda de colisión dando lugar a sus respectivos iones producto o fragmentos. Este modo de trabajo posibilita la adquisición simultánea del ion molecular (protonado o desprotonado, en función del modo de ionización seleccionado) y de los iones obtenidos tras su fragmentación en una sola inyección. Por norma general, en los espectros a baja energía de colisión predominan los iones no fragmentados de las moléculas precursoras (y en ocasiones, los aductos) mientras que a alta energía abundan los iones producto. En ocasiones, en el espectro de masas a alta energía sigue presente la información de la molécula (des)protonada. Además, MS^E proporciona información del *pattern* isotópico de los fragmentos y conserva la información de los aductos o de los dímeros. Usando este modo de trabajo, el QTOF se utiliza en modo TOF, pero se promueve la fragmentación en la celda de colisión en la función a alta energía.

Aunque podría parecer que MS^E equivale a trabajar en modo MS/MS, existen ciertas diferencias. En MS/MS se selecciona un ion de una determinada m/z, se fragmenta en la celda de colisión y se obtiene un barrido de sus iones producto con masa exacta. Este modo de trabajo permite confirmar inequívocamente la presencia de un determinado compuesto. En cambio, para realizar un *screening* rápido y de amplio rango su aplicación es limitada pues se requiere la preselección de cada ion precursor en el cuadrupolo que actúa como filtro de iones y la posterior adquisición del espectro completo de sus iones producto. Se necesitan, por tanto, dos inyecciones. Recordemos

que trabajando en el modo MS^E la información sobre la molécula (des)protonada y los iones fragmento se obtiene en la misma inyección, sin tener que seleccionar el ion precursor. Por el contrario, una de las ventajas del modo MS/MS respecto a MS^E es que en este último los componentes de la matriz entran en la celda de colisión y se fragmentan junto con los analitos. Este hecho provoca que los espectros a alta energía sean más difíciles de interpretar, pues resulta más complicado reconocer qué iones son fragmentos de un ion precursor y cuáles no. Para facilitar esta tarea se requiere una buena separación cromatográfica como la que se obtiene al trabajar con UHPLC.

Finalmente, los espectros de masas se procesan con una base de datos, que debe incluir la masa exacta teórica de la molécula neutra y (des)protonada así como la fórmula molecular de cada compuesto, y un software especializado (en nuestro caso, ChromaLynx XS, Waters) que facilita la búsqueda y confirmación de los compuestos. El software utiliza la fórmula molecular para calcular la masa exacta para $[M+H]^+$ o $[M-H]^-$, según el modo de ionización escogido. A continuación, extrae automáticamente la masa exacta de de los analitos con una ventana de masa muy estrecha (10 – 20 mDa) e indica, empleando un código de colores, los posibles candidatos encontrados en la muestra y los que no lo son (verde y rojo respectivamente), en función del error de masa. Además, muestra simultáneamente el espectro de masas completo de los posibles positivos encontrados.

La aplicabilidad del QTOF en modo MS^E para la identificación de contaminantes de distintas familias (fármacos, plaguicidas, drogas de abuso, etc.) ha quedado demostrada en varios trabajos de nuestro grupo de investigación (Hernández, 2011-b; Díaz, 2012).

Existe otra aproximación denominada *Data Dependent Acquisition* (DDA) que también proporciona información estructural en una sola inyección. En primer lugar, se adquiere el espectro en modo *full scan*, a continuación se procesa el mismo para determinar los candidatos de interés en función de unos criterios de selección previamente definidos. Si se cumplen estos criterios se realiza un barrido de iones productos seleccionando automáticamente como iones precursores los iones que

cumplieron los criterios, obteniendo información MS/MS muy útil para identificar/confirmar los compuestos seleccionados Esta aproximación resulta especialmente adecuada para el análisis de compuestos desconocidos. Sin embargo, la principal desventaja reside en el éxito de los criterios de selección escogidos para identificar un ion de la muestra como relevante para adquirir su espectro MS/MS. Desafortunadamente, en análisis ambiental los niveles de los compuestos de interés suelen ser muy bajos, por lo que la abundancia de los iones no suele dar buenos resultados como criterio de selección.

A continuación se presentan dos artículos científicos en los que se ha hecho uso de un analizador híbrido QTOF en modo MS^E combinado con UHPLC para estudiar la presencia de metabolitos de fármacos en aguas.

En el primero de ellos se ha realizado un análisis retrospectivo de muestras de efluente en las que previamente se habían detectado diversos fármacos. Se investigaron aproximadamente 160 metabolitos y de ellos se identificaron cinco. La lista de los metabolitos estudiados se confeccionó en base a varios criterios:

- Metabolitos de los fármacos estudiados en el *artículo científico 2*, reportados en la bibliografía
- Metabolitos de los fármacos incluidos en el *artículo científico 2* cuyo patrón podía adquirirse comercialmente
- Metabolitos que habían sido detectados en las aguas en trabajos publicados por otros autores

En el segundo artículo se realiza un estudio sobre los metabolitos de un analgésico muy consumido llamado dipirona (Nolotil), que nos permitió resaltar los beneficios que proporciona la espectrometría de masas de alta resolución y la importancia que tiene la separación cromatográfica en situaciones problemáticas en las que metabolitos de un compuesto pueden compartir los mismos fragmentos.

La elección de la dipirona no se produjo al azar sino que este estudio partió de una situación problemática observada en un trabajo anterior (en concreto, el método multirresidual mediante UHPLC-MS/MS (QqQ) presentado en el *artículo científico 2*). Se observó que, en ocasiones, el tiempo de retención de uno de los analitos analizados (4-aminoantipirina, metabolito de la dipirona) cambiaba notablemente. Además, el pico cromatográfico de las dos transiciones seleccionadas mostraba un hombro. En un principio, ninguno de estos hechos suponía un problema ya que tanto la desviación en el *ion ratio* como en el tiempo de retención respecto a un patrón de referencia cumplían las tolerancias establecidas. Sin embargo, decidimos investigar si los hechos observados se debían a la elevada carga de matriz o bien a la presencia de interferentes con las mismas propiedades espectrométricas que el analito (interferentes isobáricos).

3.2.2 Artículo científico 5

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Research Article

Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater

The presence of pharmaceuticals in the environment is a matter of major concern because of their wide consumption and their potential negative effect on the water quality and living organisms. After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form as the parent compound and/or as free or conjugated metabolites. These compounds seem not to be completely removed during wastewater treatments and might finally arrive to surface and ground waters. Consequently, both parent pharmaceuticals and metabolites are target analytes to be considered in analytical methodologies. The satisfactory sensitivity in full-acquisition mode, high-resolution, exact mass measurements and MS/MS capabilities of hybrid quadrupole time-of-flight (QTOF) mass spectrometry make of this technique a powerful analytical tool for the identification of organic contaminants. In this study, the use of QTOF-MS with the aid of specialised processing-data application managers has allowed the retrospective analysis of pharmaceuticals metabolites in urban wastewater without the need for additional injection of sample extracts. Around 160 metabolites have been investigated in wastewater samples previously analysed only for parent compounds using LC-QTOF under MS^E mode (simultaneous recording of two acquisition functions, at low and high collision energy). The retrospective analysis was applied to search for pharmaceutical metabolites in parent-positive effluent wastewaters from the Spanish Mediterranean region. Five metabolites, such as clopidogrel carboxylic acid or *N*-desmethyl clarithromycin, were identified in the samples.

Keywords: MS^E / Pharmaceutical metabolites / Retrospective analysis / Time-of-flight mass spectrometry / Ultrahigh-pressure liquid chromatography / Urban wastewater
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1 Introduction

In last few years, there has been an increasing concern over the presence of emerging contaminants in aquatic ecosystems [1, 2]. Similar to other emerging pollutants, like illicit drugs, personal care products, or pesticide metabolites, pharmaceuticals have received much attention since their presence was reported in surface water and urban wastewater [3, 4]. However, with few exceptions [5], much less attention has been paid to pharmaceutical metabolites.

After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form as the parent compound, but also being transformed to one or more metabolites and excreted as a mixture. Several of these compounds are not completely removed during wastewater treatments and they may eventually reach surface and ground waters [6–8].

There is some information about possible human and ecological adverse effects derived from the presence of pharmaceuticals in the aquatic ecosystem. The low concentration levels typically found in environmental water seem not to cause toxic effects, but few data are available about long-term risk derived from their continuous input in the aquatic environment. In the case of metabolites, information about their fate, occurrence and effects in the environment is still scarce. However, they should be taken into account because the exposure to metabolites may also have hazardous effects similar to those of the parent compound [8, 9].

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Abbreviations: **HE**, high energy; **LE**, low energy; **XIC**, eXtracted ion Chromatograms

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Retrospective LC-QTOF MS analysis searching for pharmaceutical metabolites in urban wastewater

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ABSTRACT

The presence of pharmaceuticals in the environment is a matter of major concern because of their wide consumption and their potential negative effect on the water quality and living organisms. After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form as the parent compound and/or as free or conjugated metabolites. These compounds seem not to be completely removed during wastewater treatments and might finally arrive to surface and ground waters. Consequently, both parent pharmaceuticals and metabolites are target analytes to be considered in analytical methodologies. The satisfactory sensitivity in full-acquisition mode, high-resolution, exact mass measurements and MS/MS capabilities of hybrid quadrupole time-of-flight (QTOF) mass spectrometry make of this technique a powerful analytical tool for the identification of organic contaminants. In this study, the use of QTOF-MS with the aid of specialised processing-data application managers has allowed the retrospective analysis of pharmaceuticals metabolites in urban wastewater without the need for additional injection of sample extracts. Around 160 metabolites have been investigated in wastewater samples previously analysed only for parent compounds using LC-QTOF under MS^E mode (simultaneous recording of two acquisition functions, at low and high collision energy). The retrospective analysis was applied to search for pharmaceutical metabolites in parent-positive effluent wastewaters from the Spanish Mediterranean region. Five metabolites, such as clopidogrel carboxylic acid or *N*-desmethyl clarithromycin, were identified in the samples.

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Most of the recent methods for determination of pharmaceuticals in the environment are based on LC-MS/MS using triple quadrupole (QqQ) analyzer, where the analytes need to be selected before MS-data acquisition [10]. The excellent sensitivity and selectivity achieved in selective reaction monitoring (SRM) mode are widely recognized, making this technique very attractive for quantification and identification of pharmaceuticals in natural and wastewater.

However, using this approach other potential contaminants not included in the method would not be detected, even if they were present at high levels in the sample.

The trend when using LC-MS/MS QqQ is towards the simultaneous multi-class determination of pharmaceuticals, including from a few to 50–70 target analytes [10-13]. Most of these target methods are focused on parent compounds while metabolites have scarcely been taken into account. Several reasons can explain the absence of analytical methodology for metabolites: first, the relevance of metabolites in the environment has not much been considered by researchers until now possibly due to the lack of information (although data about metabolites and excretion rates should exist in the market authorization files according to the European Medicine Agency (EMEA) [14] (www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/10/WC500003978.pdf), which establishes the need for evaluating potential risks of pharmaceutical products to the environment, this information is difficult to obtain). Another reason is the lack of commercially available reference standards. In addition, the quite different physico-chemical characteristics of pharmaceutical metabolites in comparison to the parent compounds complicate their simultaneous extraction/determination. Metabolites are highly polar and commonly poorly retained on reversed-phase LC columns [15]. This may also be problematic in the solid-phase extraction step (SPE). As a consequence, metabolites have hardly been studied in targeted analysis. Typically, only the metabolites of those compounds classified as pro-drugs, such us dipyrone or acetylsalicylic acid, have been included in these methods [16, 17]. Recently, Tarcomnicu et al. [5] developed an LC-MS/MS method for the analysis of 15 top-sold prescription pharmaceuticals and some of their important metabolites in influent wastewater. After analysis of 21 wastewater samples, all target compounds were detected although at concentrations below the limit of quantification (0.5–25 ng/L).

The sensitive full-spectrum accurate mass data provided by time of flight (TOF) MS are especially suited for wide-scope screening and identification of organic pollutants in environment. TOF technology offers the possibility of investigating the presence of compounds once the analysis has been performed and MS data acquired (post-target analysis) [18]. Compounds to be searched do not need to be selected before analysis, because information acquired is not analyte specific, but general information contained in the full accurate-mass spectrum. The advantage of this post-target approach is the ability to detect and identify other compounds of interest without performing additional analyses (i.e. retrospective analysis). Using

this approach hundreds, even thousands, of compounds might be, in principle, investigated [19-21], in contrast to QqQ methods.

In the last years, there has been a growing trend on using TOF instruments for screening and confirmation of pharmaceuticals in environmental samples [22-25]. In all these cases, TOF has been proven to be an efficient approach for confirmation of compounds. However, despite its excellent potential for screening and identification purposes, it has been scarcely used for the study of pharmaceutical metabolites.

In our laboratory, hybrid quadrupole-TOF (QTOF) MS analyzer combined with UHPLC was used for wide-scope screening of emerging contaminants in urban wastewater. Analyses were made in MS^E mode, which involves recording two functions at different collision energies: low energy (LE), where none or poor fragmentation takes place, and high energy (HE), where fragmentation in the collision cell is promoted. MS^E approach enables the simultaneous acquisition of both the (de)protonated molecule and fragment ions in a single acquisition. Full-spectra acquisition data obtained are processed using specialized software, allowing searching for compounds of interest without an additional analysis. This is an excellent approach for screening and identification purposes [19, 21, 26, 27].

The goal of this work is to investigate the presence of pharmaceutical metabolites in the water samples previously analyzed by UHPLC-QTOF-MS, using a retrospective analysis strategy. Around 160 metabolites have been investigated in effluent wastewater samples previously analysed only for parent compounds.

2. Materials and methods

2.1. Reagents and chemicals

Reference standards of pharmaceuticals (see Table 1 in Supporting Information), and two pharmaceutical metabolites (4-aminoantipyrine and salicylic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA), LGC Promochem (London, UK), Toronto Research Chemicals, Across Organics (Geel, Belgium), Bayer Hispania (Barcelona, Spain), Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid, Spain) and Aventis Pharma (Madrid, Spain). All reference standards presented purity higher than 93%.

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), Sodium hydroxide >99% (NaOH), ammonia solution (25%) and formic acid (98–100%) were acquired from Scharlau (Barcelona, Spain). Leucine enkephalin, used as the lock mass and imazalil, used for calibration, were purchased from Sigma-Aldrich and Dr. Ehrenstorfer (Augsburg, Germany), respectively.

2.2. Instrumentation

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (Q-oaTOF Premier, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI interface operating in positive- and negative-ion modes. The UHPLC separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size analytical column 100×2.1 mm (Waters) at a flow rate of 300 µL/min. The mobile phases used were A=H_2O with 0.01% HCOOH and B=MeOH with 0.01% HCOOH. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10%; 14 min, 90%; 16 min, 90%; 16.01 min, 10%; 18 min, 10%.

For MS^E experiments, two acquisition functions with different collision energies were created: the LE function selecting a collision energy of 4 eV, and the HE function, with a collision energy ramp ranging from 15 to 40 eV.

Further details on instrument operating conditions, both chromatographic and spectrometric, can be found elsewhere [21].

2.3. Water samples

Twenty-four parent-positive effluent wastewaters from the Spanish Mediterranean region were re-examined to investigate the presence of pharmaceutical metabolites. These water samples had been previously analysed by UHPLC-(Q)TOF-MS, after SPE. Briefly, 100 mL water samples were passed through Oasis HLB (60 mg) cartridges, previously conditioned with 3 mL MeOH and 3 mL HPLC-grade water. After drying under vacuum, analytes were eluted with 5 mL methanol. The extract was evaporated to dryness under a gentle nitrogen stream at 40℃ and reconstituted with 1 mL MeOH–water (10:90, v/v).

3. Results and discussion

3.1. Initial screening of pharmaceuticals in urban water samples

Different water samples had been previously analysed by UHPLC-(Q)TOF-MS using MS^E approach [19, 21, 27]. By applying LE in the collision cell (typically 4 eV), fragmentation is minimized and the information obtained corresponds normally to non-fragmented ions related to the parent molecule (adducts in some cases). On the contrary, at high collision energy (HE), fragmentation of the molecule is favoured, resulting in more abundant fragments. MS^E provides not only fragmentation spectra similar to those of the MS/MS experiments, but also the isotopic pattern of the fragments, and it conserves adduct and/or dimmer information. With MS^E experiments, both (de)protonated molecule and fragment ion data are enabled in a single acquisition, without the need for selecting the precursor ion, a notable difference with true MS/MS experiments.

Full-spectrum accurate mass, generated simultaneously at low and high collision energies, were later processed using specialised software in a targeted mode, allowing less time consuming, more efficient and automated process. ChromaLynx XS in the target way (software provided by Waters) offers the possibility of applying a “post-target” processing method based on monitoring exact masses of the selected analytes using narrow mass windows (e.g. 10–20 mDa) that permits a rapid and simple reviewing by cataloguing analytes, as a function of mass error. In addition, this software allows the simultaneous visualization of the complete spectrum of positive findings. Around 350 pharmaceuticals had been included in the initial database. As 77 reference standards were available at our laboratory, experimental data (retention time and fragment ions) were helpful for rapid detection and more confident identification. For the remaining compounds, only the theoretical mass and molecular formulae were included in the database. Identification of compounds was based on accurate masses of the (de)protonated molecule and of their fragment ions, compatibility of the fragments chemical structures (manually and/or with the help of MassFragment software) with the suspected compound, coincidence of retention times for all eXtracted ion Chromatograms (XICs) (typically $[M+H]^+$ and more abundant fragment ions) and isotopic fit (experimental versus theoretical isotopic pattern). Finally, when available information strongly supported the identity of the suspected compound, the reference standard should be purchased and injected for final confirmation.

The most frequently detected compounds were the antibiotics ciprofloxacin, clarithromycin, norfloxacin and ofloxacin, as well as the angiostensin II receptor antagonists valsartan and irbesartan, used in the treatment of hypertension. The antidepressant (http://en.wikipedia.org/wiki/Antidepressant) venlafaxine, the loop diuretic furosemide, the non-steroidal anti-inflammatory drugs diclofenac and ibuprofen were also widely found (http://en.wikipedia.org/wiki/Non-steroidal_anti-inflammatory_drug).

3.2. Retrospective analysis of parent-pharmaceutical positive water samples

Oppositely to those analytical methods, where analyte-specific information is required before injecting a sample, e.g. methods based on selected ion monitoring (SIM) or SRM, accurate-mass full-spectrum data generated by LC-(Q)TOF-MS remain available over time, even when dealing with target analysis. Thus, a retrospective analysis is always feasible and, in principle, any compound could be investigated in a post-target way provided such compound has passed the sample preparation, chromatographic separation and ionization process with sufficient efficiency. This fact, clearly represents an important advantage of high-resolution MS.

Data can be reprocessed and re-evaluated using new or modified databases to search for other relevant compounds, that were not considered in the first analysis, simply by including their theoretical exact mass and molecular formulae into the database. A new compound database was compiled in this work (see Table 2 in Supporting Information), including around 160 theoretical exact masses of metabolites, derived from 50 parent pharmaceuticals. Analytes were selected based on several criteria: those metabolites reported in the literature for the pharmaceuticals included in our previous LC-MS/MS method [21]; lists of commercially available metabolite reference standards of pharmaceuticals included in [21]; metabolites detected in water samples by other authors.

Full-spectrum MS data generated at LE were processed using the ChromaLynx XS in a targeted mode. In a first step, the presence of the metabolite ion (typically $[M+H]^+$ or $[M-H]^-$) measured at its accurate mass (nw-XIC of 0.02 Da) was evaluated. In the case that a chromatographic peak was observed, the suspected positive was investigated by evaluating the mass error of the (de)protonated molecule together with its characteristic isotopic distribution. The fragment ions, in both LE and HE spectra, were also studied. Different strategies were

applied to improve the confidence in the identification, as metabolites reference standards were not available at our laboratory.

When the accurate-mass fragment ions of the parent pharmaceutical were known because the reference standard was available at our laboratory, we could predict the accurate-mass fragments of the suspected metabolite taking into account the structural differences between both molecules. When fragmentation of the parent pharmaceutical was not available in our empirical database, accurate mass fragments were proposed using specialized software (e.g. MassFragment). In this case, information about product ions reported in the literature for the suspected metabolite or for the parent compound was essential. This information could come from analysis based on nominal mass measurement (e.g. LC-MS/MS with QqQ) or accurate mass measurements (e.g. LC-(Q)TOF-MS). Next, some examples illustrating the strategies followed in this work are shown.

3.2.1 Fragment ions of the parent pharmaceutical available in the empirical database

As an example, Fig. 1 shows the detection and identification of *N*-desmethyl-clarithromycin, a metabolite of the antibiotic clarithromycin, in effluent wastewater. After automatically performing an nw-XIC at *m/z* 734.4710, corresponding to protonated *N*-desmethyl-clarithromycin ($C_{37}H_{68}NO_{13}$), a chromatographic peak was depicted at 9.59 min (Fig. 1D, bottom), very close to the retention time of the parent clarithromycin ($[M+H]^+$: $C_{38}H_{70}NO_{13}$, *m/z* 748.4847, retention time 9.58 min, Fig. 1C bottom), which was also detected in the sample. Clarithromycin presents two main fragment ions in the HE mass spectra at *m/z* 590.3904 ($C_{30}H_{56}NO_{10}$) and 158.1181 ($C_8H_{16}NO_2$) (Fig.1C, top and middle). According to fragmentation shown in Fig. 1A for clarithromycin and, taking into account the position in which the demethylation occurs (Fig. 1B), the expected elemental compositions for the corresponding metabolite fragment ions would be $C_{29}H_{54}NO_{10}$ (*m/z* 576.3748) and $C_7H_{14}NO_2$ (*m/z* 144.1025). As Fig. 1D shows, chromatographic peaks for both ions were obtained at exactly the same retention time, strongly supporting the identity of this suspected compound. In this case, no information about LC-MS/MS behaviour of this metabolite was found in the literature, which avoided to further advance in the confirmation of the identity of this metabolite. At this point, the reference standard was acquired and injected, confirming the identity of the metabolite. This metabolite was found in 3 of the 24 samples analysed (around 13%).

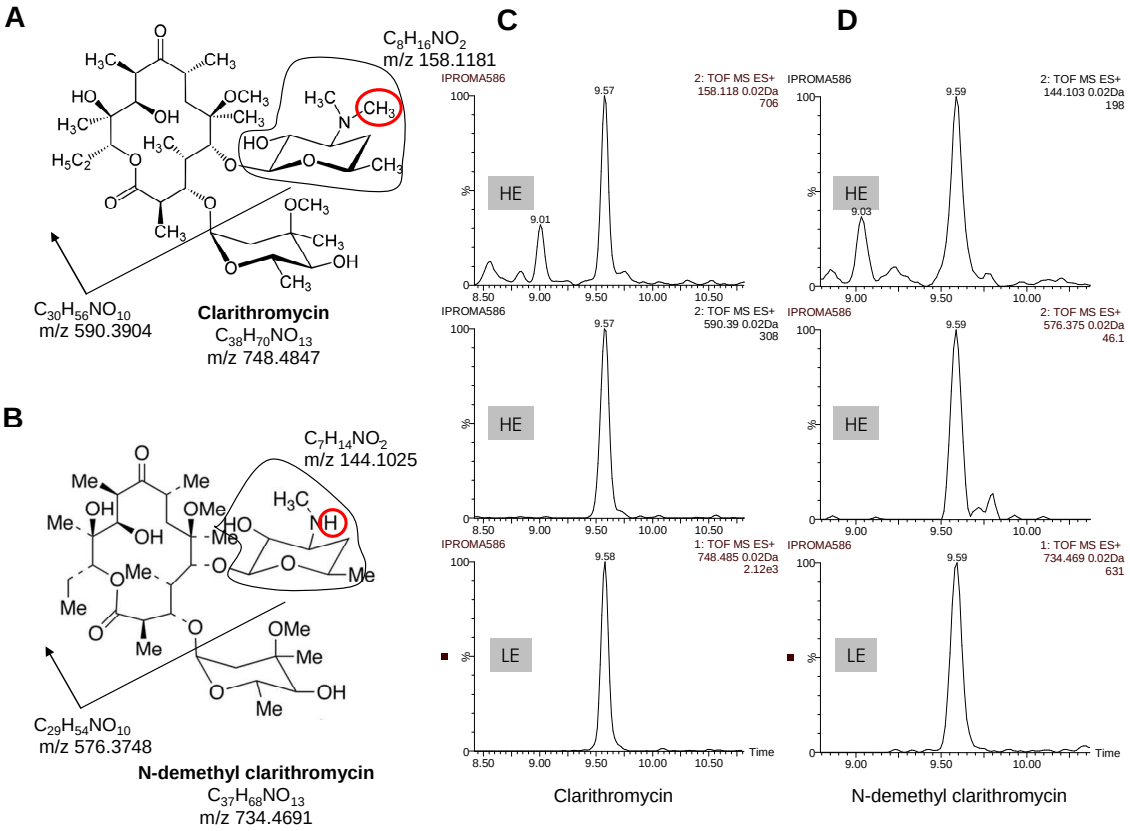

Figure 1. Detection and identification of *N*-desmethyl clarithromycin in effluent wastewater by UHPLC-QTOF-MS (MS^E approach). (A) Structure of antibiotic claritromycin and main fragment ions; (B) Structure of *N*-desmethyl claritromycin and proposed fragment ions; (C) and (D) XICs at 20 mDa mass window for $[M+H]^+$ in LE function and two fragments in HE function for parent clarithromycin and its metabolite, respectively.

Figure 2 illustrates the detection and identification of another metabolite of clarithromycin, 14-hydroxy-clarithromycin, in an effluent wastewater. After performing an nw-XIC at *m/z* 764.4796, corresponding to 14-hydroxy-clarithromycin ($C_{38}H_{70}NO_{14}$), a chromatographic peak was observed at 8.04 min (Fig. 2D, bottom) in the LE function. In agreement with the two main fragment ions, *m/z* 590.3904 ($C_{30}H_{56}NO_{10}$) and 158.1180 ($C_8H_{16}NO_2$), and the fragmentation shown for clarithromycin in Fig. 2A, and taking into account the position in which the hydroxylation occurs (Fig. 2B), the expected elemental composition for the metabolite fragment would be $C_{30}H_{56}NO_{11}$, with *m/z* 606.3853. On the other hand, the fragment at *m/z* 158.1181 ($C_8H_{16}NO_2$) would be common for both compounds (Fig. 2A and B). When performing

the XICs at the expected exact masses, a chromatographic peak was observed at all *m/z* values at the same retention time, strongly supporting the suggested identity of this compound. In this case, the ion at *m/z* 606 observed in the HE TOF spectrum was in accordance with the product ion reported in a QqQ method previously reported [28]. In a strict sense, this behaviour would also match with other hydroxylated metabolites, not only with that suggested in the position 14. However, as the 14-hydroxy is the main metabolite reported in the literature for claritromycin, it seems reasonable to believe this is the compound detected in wastewater. Obviously, the reference standard should be acquired and injected for unequivocal confirmation. However, in this case, it was not commercially available. This metabolite was detected in 5 of the 24 samples analysed (around 21%).

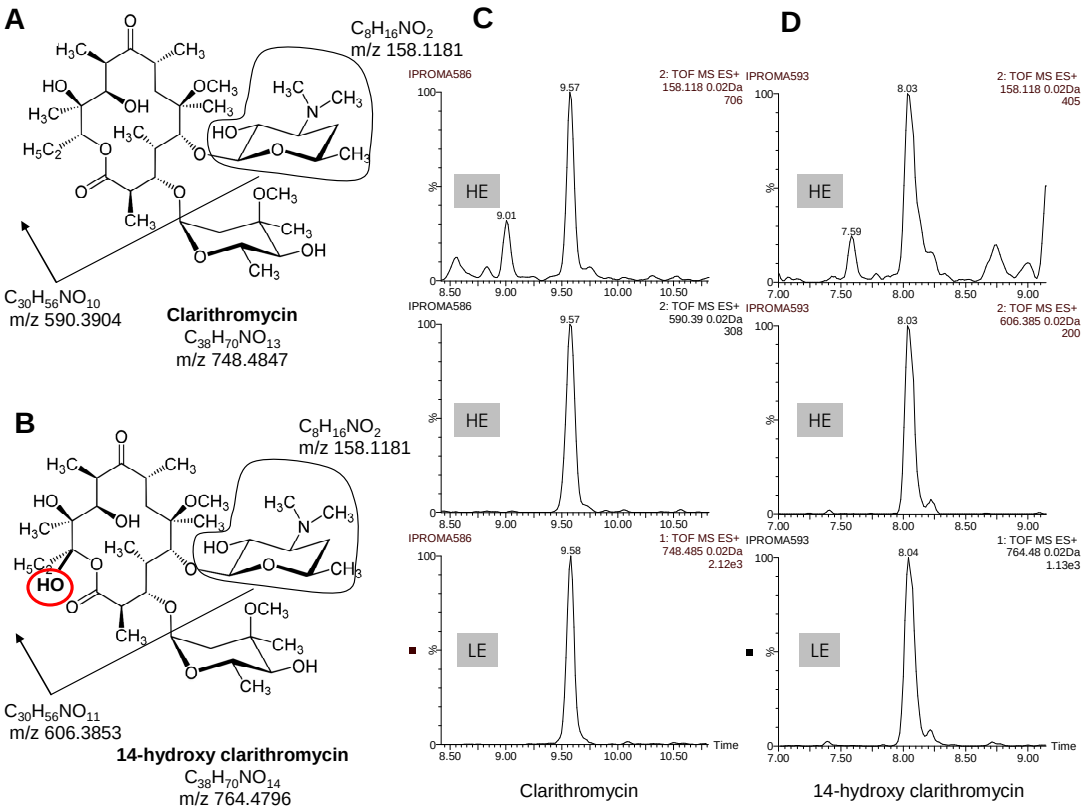

Figure 2. Detection and identification of 14-hydroxy clarithromycin in effluent wastewater by UHPLC-QTOF-MS (MS^E approach). (A) Structure of antibiotic claritromycin and main fragment ions; (B) Structure of 14-hydroxy claritromycin and proposed fragment ions; (C) and (D) XICs at 20 mDa mass window for $[M+H]^+$ in LE function and two fragments in HE function for parent claritromycin and its metabolite, respectively.

Another illustrative example is the detection and identification of a metabolite of omeprazole, one of the most widely prescribed drugs (Fig.3). Despite its wide consumption, this compound is seldom detected in water, as it is almost completely metabolized by the cytochrome P450 system, mainly in the liver. Some of the main metabolites reported are hydroxy-omeprazole, a sulphide derivative and a sulphone [29].

As can be seen in Fig.3, two chromatographic peaks were obtained at *m/z* 316.1120 in the LE function (5.55 and 6.03 min), which corresponds to the protonated molecule of either 4-desmethoxy omeprazole, 4-hydroxy-omeprazole sulphide or 5-*O*-desmethyl omeprazole sulphide, all sharing the same elemental composition ($C_{16}H_{18}N_3O_2S$). Omeprazole ($C_{17}H_{20}N_3O_3S$, *m/z* 346.1225) presents a main fragment ion at *m/z* 198.0589 ($C_9H_{12}NO_2S$). Another fragment is observed at *m/z* 150.0920 ($C_9H_{12}NO$), although with low abundance.

According to the fragmentation shown for omeprazole in Fig. 3, and taking into account the position where the metabolism occurs, the expected elemental compositions would be $C_8H_{10}OS$ (*m/z* 168.0483), for 4-desmethoxy omeprazole and 4-hydroxy-omeprazole sulphide, and $C_9H_{12}NOS$ (*m/z* 182.0640) for 5-*O*-desmethyl omeprazole sulphide. The nw-XIC at *m/z* 168.0483, showed the presence of a peak at 6.04 min. However, no peak was observed for *m/z* 182.0640, discarding that 5-*O*-desmethyl omeprazole sulphide was the metabolite detected in water.

At this point, more information was obtained from the other fragment ion (*m/z* 150.0920) of the parent omeprazole. The expected elemental composition for the corresponding metabolite fragments would be $C_8H_{10}NO$ (*m/z* 136.0762) for 4-hydroxy-omeprazole sulphide and $C_8H_{10}N$ (*m/z* 120.0813) for 4-desmethoxy omeprazole. However, this case no chromatographic peaks were observed at these *m/z* values, which might be due to the low abundance of this ion. In the absence of more information, reference standards should be acquired and injected to discard between the two remaining candidates. This compound was detected in the 54% of the samples analysed (13/24).

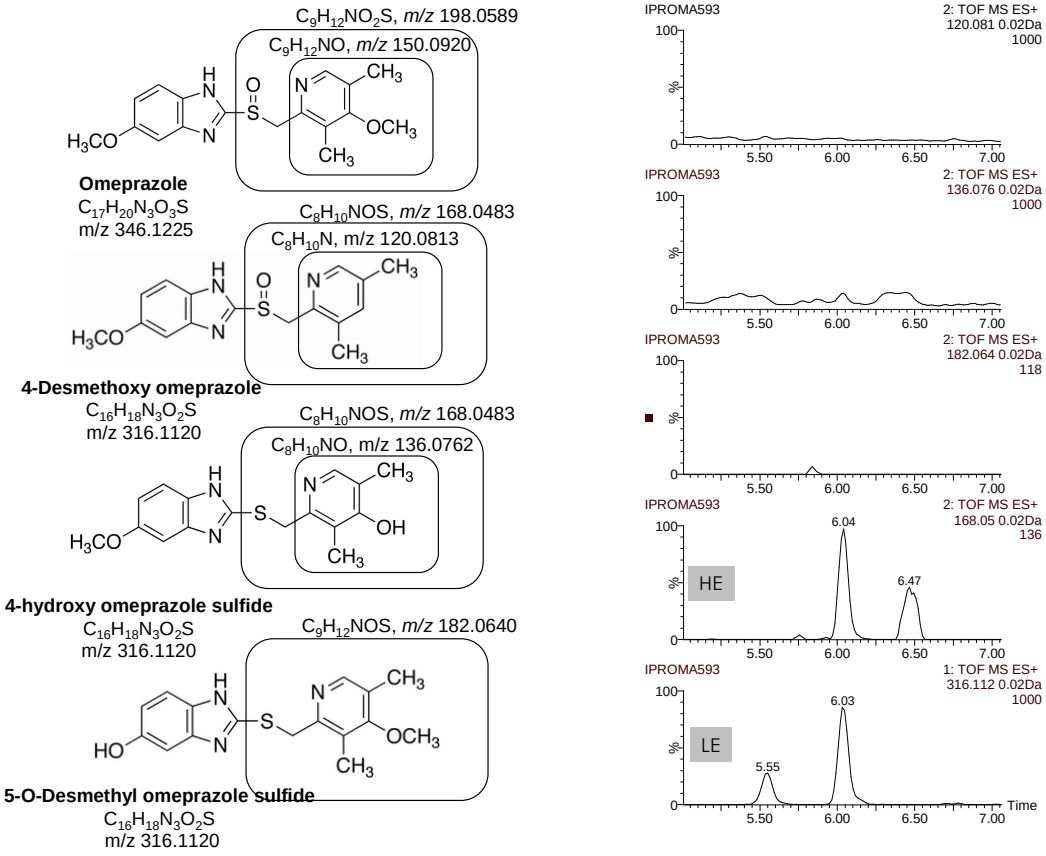

Figure 3. Detection and identification of an omeprazole metabolite in effluent wastewater by UHPLC-QTOF-MS (MS^E approach). Left: Structures of omeprazole and metabolites, together with proposed fragment ions. Right: XICs at 20 mDa mass window for different ions.

3.2.2 Fragment ions of the parent pharmaceutical was not available in the empirical database

When the reference standard of the parent pharmaceutical was not available, the strategy to improve the confidence in the identification consisted on justifying accurate mass fragments using specialized software (MassFragment, from Waters). This software applies a bond-disconnecting methodology to obtain possible structures for the fragment ions from a given molecule. After that, the main fragments of the suspected metabolite were compared with

product ions reported in the literature for the candidate, if available, and/or for the parent pharmaceutical.

Figure 4 shows the detection and identification of clopidogrel carboxylic acid in an effluent wastewater sample. Clopidogrel is an oral, thienopyridine class antiplatelet agent used to inhibit blood clots in coronary artery disease, peripheral vascular disease and cerebrovascular disease. The accurate mass of the protonated molecule of the suspected clopidogrel carboxylic acid, retention time 5.64 min, was found to be *m/z* 308.0524 from the LE MS spectrum (Fig. 4B, bottom). The mass error with respect the exact mass of this metabolite was 1.2 mDa. The combined spectrum for this chromatographic peak showed a typical monochlorinated isotopic pattern, in accordance with the elemental composition of the protonated molecule of this metabolite ($C_{15}H_{15}NO_2SCl$). The HE TOF-MS spectrum was also investigated (Fig. 4B, top). Up to 7 fragment ions, all showing the Cl isotopic pattern were obtained. A possible concern of the MS^E approach is that co-eluting compounds would "contaminate" HE spectrum, which might also contain ions unrelated to the analyte, complicating the spectrum interpretation. Recognizing which ions are fragments, and which are not, becomes mandatory. At this point, UHPLC turned valuable for differentiating co-eluting ions, given the excellent resolving power of this technique. As Fig. 4C shows all XICs performed, for the protonated molecule and for the seven main fragment ions led to a chromatographic peak at the same retention time (5.63 min).

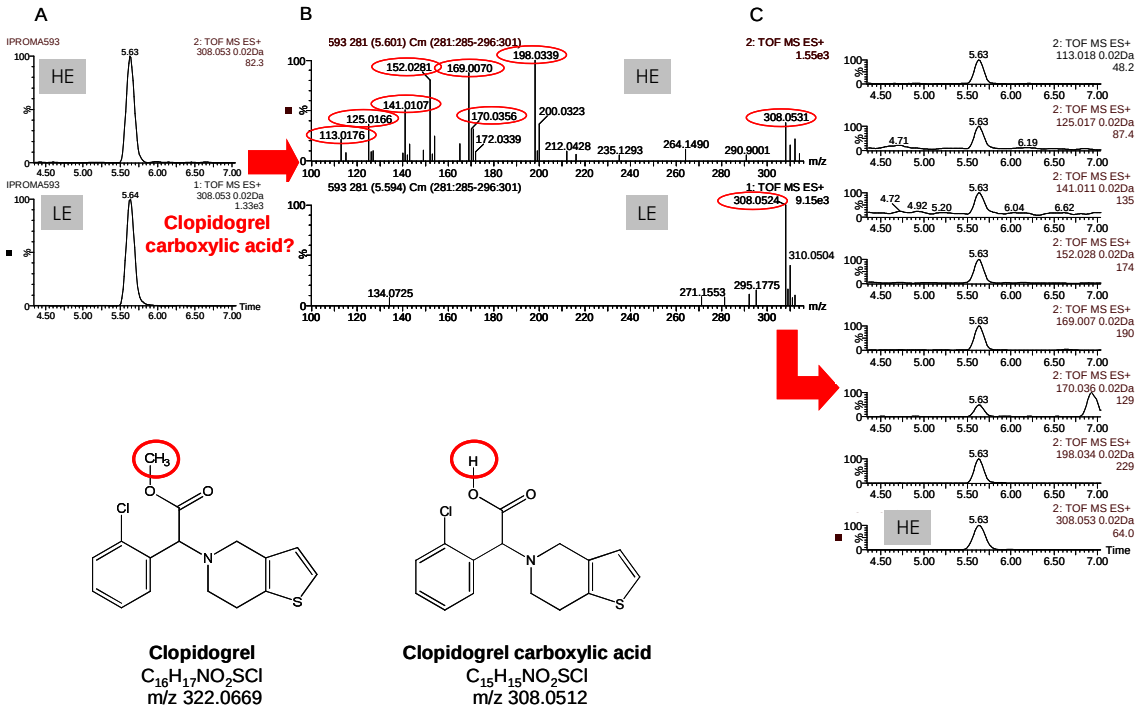

Figure 4. Detection and identification of clopidogrel carboxylic acid in effluent wastewater by UHPLC-QTOF-MS (MS^E approach). Chromatograms (A) and spectra (B) of the sample; (C) XICs at 20 mDa mass window for different ions observed in HE function.

Reliable elemental composition for all fragments was calculated, obtaining errors for experimental accurate masses normally below 2 mDa in relation to the theoretical masses predicted. Figure 5 shows the structure of the 7 fragments suggested by MassFragment software. In this case, the ions at *m/z* 198 and 152 observed in the HE TOF spectrum were in accordance with those reported in the bibliography for the metabolite 5. After this careful evaluation process, there were strong evidences supporting that clopidogrel carboxylic acid was the compound detected. Then, we acquired the reference standard, unequivocally confirming that the compound detected was that metabolite, actually. This metabolite was detected in 19 of the 24 samples analysed (around 79%).

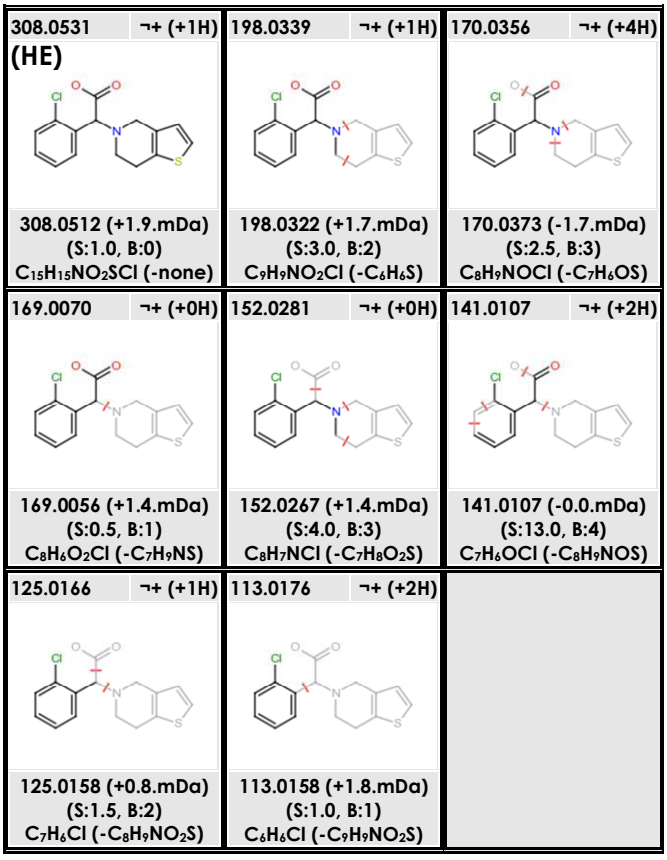

Figure 5. Structures proposed by MassFragment software for fragment ions of clopidogrel carboxylic acid.

In a similar way, the metabolite fenofibric acid was also identified in one of the samples (Fig. 6), supporting the usefulness of this approach, which was found to be highly reliable for identification purposes even without using reference standards. The accurate mass of the protonated molecule of the suspected fenofibric acid (retention time 11.3 min) was found to be *m/z* 319.0754 from the LE MS spectrum (Fig.6), with mass error of 1.7 mDa in relation to its exact mass. Moreover, the combined spectra of this chromatographic peak showed the one-chlorine isotopic pattern, in accordance with the elemental composition of the protonated molecule of this metabolite ($C_{17}H_{16}O_4Cl$). Elemental composition for the four main fragments observed in the HE TOF-MS was calculated, obtaining errors for experimental accurate masses normally below 1.5 mDa in relation to the theoretical exact masses predicted. The structures suggested are also depicted in Fig. 6. The ions at *m/z* 233, 139 and 121 observed in the HE

TOF spectra matched with the product ions reported in a previous LC-MS/MS method using triple quadrupole 30, strongly supporting the identity suggested for this metabolite. This metabolite was found in 14 of the 24 samples analysed (around 58%).

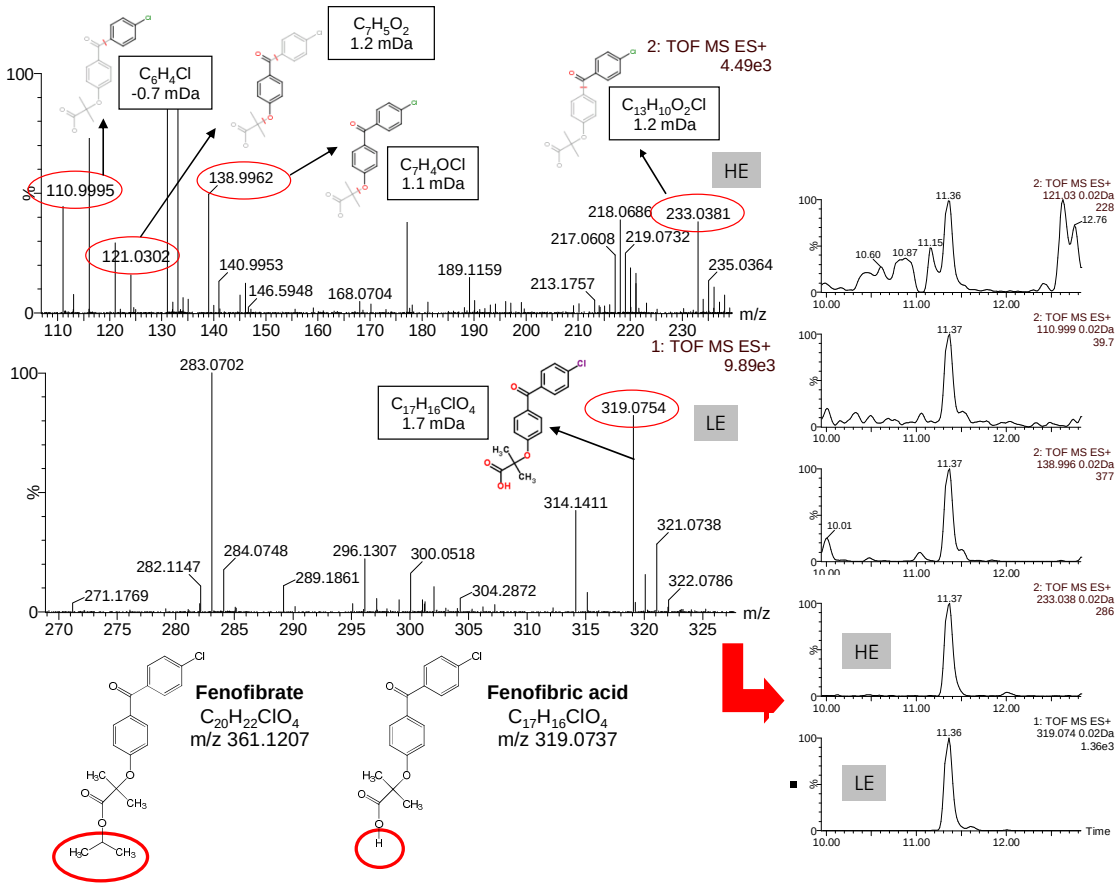

Figure 6. Detection and identification of fenofibric acid in effluent wastewater by UHPLC-QTOF-MS (MS^E approach). Left: LE and HE spectra of the sample. Structure of fenofibric acid and MassFragment justification of its most abundant fragments. Right: XICs at 20 mDa mass window for the protonated molecule in LE function and for different ions observed in HE function.

In all cases of positive metabolite findings, the empirical information obtained on fragment ions was added to our database in order to facilitate future screenings. Once the reference standards will be acquired, it would also be feasible to include retention time and experimental fragment ions to make the detection and identification easier in future works.

In our experience, analysis by LC-QTOF under MS^E mode allows a highly reliable identification of the compounds detected. Previous analyses of natural and wastewater led to the finding of several suspected pharmaceuticals (e.g. irbesartan, valsartan, furosemide) that all were subsequent confirmed when the reference standard was acquired. Therefore, using this approach, the subsequent acquisition of reference standards is made on the basis of solid evidences, avoiding the cost and inconveniency of buying all standards (some of them difficult to obtain), which should be required in most of target methods.

4. Conclusion remarks

UHPLC-QTOF-MS is a powerful technique for screening and identification of pharmaceuticals. Analysis of urban wastewater has allowed the detection and identification of several pharmaceuticals in the samples. The retrospective evaluation of the full-scan accurate-mass data has allowed, in this work, the identification of some reported-pharmaceuticals metabolites, such as *N*-desmethyl clarithromycin, 14-hydroxy-clarithromycin, fenofibric acid and clopidogrel carboxylic acid. In addition, metabolites of the widely but seldom detected in water omeprazol (4-hydroxy omeprazole sulphide or 4-desmethoxy omeprazole) have also been found. Different reasons can explain why other potential known metabolites have not been found, ranging from excretion relevance to further transformation during wastewater treatment. The use of a QTOF instrument has allowed the efficient fragmentation of the analyte molecule in the collision cell, working under TOF-MS mode. Operating the instrument in MS^E acquisition mode it is feasible to simultaneously obtain full-spectrum accurate-mass data at low and high collision energy. The combination of these two datasets is highly useful for identification and elucidation purposes, as LE MS spectra usually show the protonated molecule (in positive-ESI), while HE MS spectra are rather rich in fragment ions. With all information provided by this technique (accurate mass, isotopic distribution and MS data at LE and HE), and the efficient chromatographic separation offered by UHPLC, it is feasible to identify compounds in complex environmental matrices, by searching for target analytes (parent or metabolites) on the basis of a compound database. With a good knowledge of the technique, and after appropriate

treatment of all MS data provided, there is a high reliability in the identification of suspected candidates, even without reference standards. Ultimate confirmation would be reached by injecting reference standards, which might be acquired only in those cases where QTOF experimental data strongly support their presence in the samples. The development of MS^E spectra libraries would be highly useful for future works to facilitate this task.

Similar to the work performed in this article, the information obtained from QTOF-MS analysis could be used in the future to search for other contaminants (e.g. pesticides, drugs of abuse, UV filters,…) and/or metabolites without the need for additional analysis. Obviously, those contaminants should meet the requirements derived from sample preparation (in this work, SPE with Oasis HLB cartridges) and LC-MS/MS analysis (i.e. satisfactory chromatographic separation and efficient API ionization).

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The authors have declared no conflict of interest.

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Supplemental Information

Table 1. List of available pharmaceutical standards.

Compound	Therapeutic group	Compound	Therapeutic group
Acetaminophen	Analgesic and anti-inflammatories	Sulfamethoxazole	Sulfonamide antibiotics
4-Aminoantipyrine		Sulfamethazine	
Diclofenac		Sulfadiazine	
Ibuprofen		Sulfathiazole	
Ketoprofen		Lincomycin	Lincosamide antibiotics
Naproxen		Clindamycin	
Salicylic acid		Erithromycin	Macrolide antibiotics
Atorvastatin	Cholesterol lowering statin drugs and lipid regulators	Clarithromycin	
Simvastatin		Tylosin	
Pravastatin		Roxithromycin	
Bezafibrate		Moxifloxacin	Quinolone antibiotics
Gemfibrozil		Norfloxacin	
Paroxetine	Antidepressants	Pefloxacin	
Venlafaxine		Ofloxacin	
Omeprazole	Anti-ulcer agents	Marbofloxacin	
Pantoprazole		Ciprofloxacin	
Olanzapine	Psychiatric drugs	Enrofloxacin	
Risperidone		Sarafloxacin	
Alprazolam	Ansiolitics	Flumequine	
Lorazepam		Oxolinic acid	
Enalapril	Cardiovasculars	Nalidixic acid	
		Pipedimic acid	
		Furaltadone	Other antibiotics
		Furazolidone	
		Trimethoprim	
		Chloramphenicol	

Table 2. Compound database of pharmaceutical metabolites.

COMPOUND	ELEMENTAL COMPOSITION
Acetaminophen	C8H9NO2
3-Hydroxyacetaminophen	C8H9NO3
3-Methoxy Acetaminophen	C9H11NO3
3'-Mercaptoacetaminophen Disulfide	C16H16N2O4S2
S-Methyl-3-thioacetaminophen	C9H11NO2S
N-Acetylbenzoquinoneimine	C8H7NO2
4-Acetamidophenyl β-D-Glucuronide	C14H17NO8
4-Acetaminophen Sulfate	C8H9NO5S
3-(N-Acetyl-L-cystein-S-yl) Acetaminophen	C13H16N2O5S
3-Cysteinylacetaminophen	C11H14N2O4S
Acetylsalicylic Acid	
Salicylic acid	C7H6O3
Acetylsalicylic Acid Acyl-β-D-glucuronide	C15H16O10
Gentisic acid	C7H6O4
ortho-hydroxyhippuric	C9H9NO4
Alprazolam	C17H13ClN4
Alprazolam 5-Oxide	C17H13ClN4O
1-Hydroxy Alprazolam	C17H13ClN4O
1-Hydroxy Alprazolam β-D-Glucuronide	C23H21ClN4O7
4-Hydroxy Alprazolam Methanoate	C18H17ClN4O2
Atorvatastin	C33H35FN2O5
Atorvastatin Acetonide Acyl-β-D-glucuronide	C42H47FN2O11
2-Benzyloxy Atorvastatin Acetonide	C43H45FN2O6
2-Hydroxy Atorvastatin Acyl-β-D-glucuronide	C39H43FN2O12
2-Hydroxy Atorvastatin Lactone	C33H33FN2O5
2-Hydroxy Atorvastatin / orto-hydroxyatorvastatin	C33H35FN2O6
4-Hydroxy Atorvastatin / para-hydroxyatorvastatin	C33H35FN2O6
Bezafibrate	C19H20ClNO4
4-chlorobenzoic acid	C7H5ClO2
Carbamazepine	C15H12N2O
2-Hydroxy Carbamazepine	C15H12N2O2

COMPOUND	ELEMENTAL COMPOSITION
3-Hydroxy Carbamazepine	C15H12N2O2
Carbamazepine 10,11-Epoxide	C15H12N2O2
10,11-Dihydroxy Carbamazepine	C15H12N2O3
Chloramphenicol	C11H12Cl2N2O5
Chloramphenicol 1-O-β-D-Glucuronide	C17H20Cl2N2O11
Chloramphenicol 3-O-β-D-Glucuronide	
Ciprofloxacin	C17H18FN3O3
Ciprofloxacin N-Oxide	C17H18FN3O4
Ciprofloxacin Piperazinyl-N4-sulfate	C17H18FN3O6S
Formyl Ciprofloxacin	C18H18FN3O4
Oxociprofloxacin	C17H16FN3O4
Desethylene Ciprofloxacin	C15H16FN3O3
Clarithromycin	C38H69NO13
N-Desmethyl Clarithromycin	C37H67NO13
14-OH-clarithromycin	C38H69NO14
Clindamycin	C18H33ClN2O5S
Clindamycin Sulfoxide	C18H33ClN2O6S
Clofibrate	C12H15ClO3
Clofibric Acid	C10H11ClO3
Clofibric Acid Acyl-β-D-glucuronide	C16H19ClO9
Clopidogrel	C16H16ClNO2S
Clopidogrel Carboxylic Acid	C15H14ClNO2S
Clopidogrel Acyl-β-D-glucuronide	C21H22ClNO8S
Oxoclopidogrel	C16H16ClNO3S
N-(2,6-Dichlorophenyl)anthranilic Acid	C13H9Cl2NO2
Diazepam	C16H13ClN2O
p-Hydroxy Diazepam	C16H13ClN2O2
Diclofenac	C14H11Cl2NO2
Diclofenac Acyl-β-D-glucuronide	C20H19Cl2NO8
4'-Hydroxy Diclofenac	C14H11Cl2NO3
5-Hydroxy Diclofenac	C14H11Cl2NO3
1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one	C14H10NOCl2

COMPOUND	ELEMENTAL COMPOSITION
Dypirone	C13H17N3O4S
4-methylaminoantipyrine (4MAA)	C12H15N3O
4-formylaminoantipyrine (FAA)	C12H13N3O2
4-aminoantipyrine (AA)	C11H13N3O
4-dimethylaminoantipyrine	C13H17N3O
4-acetylaminoantipyrine	C13H15N3O2
Enalapril	C20H28N2O5
Enalaprilat Dihydrate	C18H28N2O7
Enalaprilat	C18H24N2O5
Erythromycin	C37H67NO13
N-Demethyl Erythromycin A	C36H65NO13
Fenofibrate	C20H21ClO4
Dihydro Fenofibrate	C20H23ClO4
Fenofibric Acid	C17H15ClO4
Fenofibric Acid Acyl-β-D-glucuronide	C23H23ClO10
Fenirofibrate	C17H17ClO4
Fluoxetine	C17H18F3NO
Norfluoxetine	C16H16F3NO
Flurazepam	C21H23ClFN3O
nor-Flurazepam	C15H10ClFN2O
N1-(2-Hydroxyethyl) Flurazepam	C17H14ClFN2O2
Didesethyl Flurazepam	C17H15ClFN3O
Furosemide	C12H11ClN2O5S
Furosemide Acyl-β-D-glucuronide	C18H19ClN2O11S
Saluamine	C7H7ClN2O4S
Gemfibrozil	C15H22O3
Gemfibrozil 1-O-β-Glucuronide	C21H30O9
Ibuprofen	C13H18O2
2-Hydroxy Ibuprofen	C13H18O3
Ibuprofen Carboxylic Acid / Carboxy-ibuprofen	C13H16O4
Ibuprofen acyl-β-D-glucuronide	C19H26O8
Carboxy-hydratropic acid	C10H10O4

COMPOUND	ELEMENTAL COMPOSITION
Indomethacin	$C_{19}H_{16}ClNO_4$
N-Deschlorobenzoyl Indomethacin	$C_{12}H_{13}NO_3$
O-Desmethyl-N-deschlorobenzoyl Indomethacin	$C_{11}H_{11}NO_3$
O-Desmethyl Indomethacin	$C_{18}H_{14}ClNO_4$
Irbesartan	$C_{25}H_{28}N_6O$
Irbesartan N-β-D-Glucuronide	$C_{31}H_{36}N_6O_7$
Ketoprofen	$C_{16}H_{14}O_3$
Dihydro Ketoprofen	$C_{16}H_{16}O_3$
Dihydro Ketoprofen β-D-Glucuronide	$C_{22}H_{24}O_9$
Ketoprofen Acyl-β-D-glucuronide	$C_{22}H_{22}O_9$
3-(Hydroxy-carboxymethyl)hydratopic acid	$C_{11}O_5H_{12}$
3-(Keto-carboxymethyl)hydratopic acid	$C_{11}O_5H_{10}$
Loratadine	$C_{22}H_{23}ClN_2O_2$
3-Hydroxy Desloratadine	$C_{19}H_{19}ClN_2O$
3-Hydroxy Desloratadine β-D-Glucuronide	$C_{25}H_{27}ClN_2O_7$
5-Hydroxy Desloratadine	$C_{19}H_{19}ClN_2O$
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$
Lorazepam β-D-Glucuronide	$C_{21}H_{18}Cl_2N_2O_8$
Losartan	$C_{22}H_{23}N_6ClO$
Losartan Carboxaldehyde	$C_{22}H_{21}ClN_6O$
Losartan Carboxylic Acid	$C_{22}H_{21}ClN_6O_2$
Losartan Carboxylic Acid Acyl-β-D-Glucuronide	$C_{28}H_{29}ClN_6O_8$
Mefenamic acid	$C_{15}H_{15}NO_2$
3-Hydroxymethyl Mefenamic Acid	$C_{15}H_{15}NO_3$
3-Hydroxymethyl Mefenamic Acid Acyl-β-D-glucuronide	$C_{21}H_{23}NO_9$
Mefenamic Acyl-β-D-glucuronide	$C_{21}H_{23}NO_8$
3-Carboxy Mefenamic Acid	$C_{15}H_{13}NO_4$
3-Carboxy Mefenamic Acid Acyl-β-D-glucuronide	$C_{21}H_{21}NO_{10}$
Moxifloxacin	$C_{21}H_{24}FN_3O_4$
rac cis-Moxifloxacin Acyl-β-D-glucuronide	$C_{27}H_{32}FN_3O_{10}$
Moxifloxacin N-Sulfate	$C_{21}H_{24}FN_3O_7S$

COMPOUND	ELEMENTAL COMPOSITION
Naproxen	C14H14O3
O-Desmethyl Naproxen	C13H12O3
Norfloxacin	C16H18FN3O3
N-Hydroxy Norfloxacin	C16H18FN3O4
Norfloxacin N-Oxide	C16H17FN3O4
4-Oxo Norfloxacin	C16H16FN3O4
Ofloxacin	C18H20FN3O4
Ofloxacin N-Oxide	C18H20FN3O5
Desmethyl Ofloxacin	C17H18FN3O4
Olanzapine	C17H20N4S
N-Demethyl Olanzapine	C16H18N4S
2-Hydroxymethyl Olanzapine	C17H20N4OS
Olanzapine 2-Carboxaldehyde	C17H18N4OS
Omeprazole	C17H19N3O3S
4-Desmethoxy Omeprazole	C16H17N3O2S
4-Hydroxy Omeprazole Sulfide	C16H17N3O3S
5-O-Desmethyl Omeprazole Sulfide	C16H17N3O2S
Omeprazole Sulfone N-Oxide	C17H19N3O5S
5-Hydroxy Omeprazole	C17H19N3O4S
Omeprazole Acid	C17H17N3O5S
Pantoprazole	C16H15F2N3O4S
Pantoprazole Sulfone	C16H15F2N3O5S
Pantoprazole Sulfide	C16H15F2N3O3S
Pantoprazole Sulfide-β-D-glucuronide	C22H23F2N3O9S
Paroxetine	C19H20FNO3
(-)-trans-4-[4-(4'-Fluorophenyl)-3-piperidinylmethoxy]-2-methoxyphen .	C19H22FNO3
(3S-trans)-5-[[4-(4-Fluorophenyl)-3-piperidinyl]methoxy]-2-methoxyphen	C19H22FNO3
Desmethylene Paroxetine	C18H20FNO3
Pravastatin	C23H36O7
3''-Hydroxy Pravastatin	C23H36O8
3''-Hydroxy Pravastatin Lactone	C29H44O13
Pravastatin Acyl-ß-D-glucuronide	C29H44O13
3a-Hydroxy Pravastatin Lactone	C23H34O6

COMPOUND	ELEMENTAL COMPOSITION
Ranitidine	C13H22N4O3S
Ranitidine N-Oxide	C13H22N4O4S
Desmethyl Ranitidine	C12H20N4O3S
Risperidone	C23H27FN4O2
7-Hydroxy Risperidone	C23H27FN4O3
9-Hydroxy Risperidone	C23H27FN4O3
Roxithromycin	C41H76N2O15
N-Demethyl Roxithromycin	C40H74N2O15
Simvastatin	C25H38O5
6'-Hydroxymethyl Simvastatin	C25H38O6
3"-Hydroxy Simvastatin	C25H38O6
Simvastatin Acyl-β-D-glucuronide	C31H48O12
Simvatatin Hydroxy Acid	C25H40O6
Sulfadiazine	C10H10N4O2S
N-Acetyl Sulfadiazine	C12H12N4O3S
Sulfamethazine	C12H14N4O2S
N-Acetyl Sulfamethazine	C14H16N4O3S
Sulfamethoxazole	C10H11N3O3S
N-Acetyl Sulfamethoxazole	C12H13N3O4S
N-Hydroxy Sulfamethoxazole	C10H11N3O4S
4-Nitroso Sulfamethoxazole	C10H9N3O4S
4-Nitro Sulfamethoxazole	C10H9N3O5S
Sulfamethoxazole β-D-Glucuronide	16H19N3O9S
Tamoxifen	C26H29NO
(E)-4-Hydroxy-N-desmethyl Tamoxifen	C25H27NO2
(Z)-4-Hydroxy-N-desmethyl Tamoxifen	C25H27NO2
(E)-α-Hydroxy Tamoxifen	C26H29NO2
(Z)-4-Hydroxy Tamoxifen	C26H29NO2
4'-Hydroxy Tamoxifen	C26H29NO2
(E,Z)-Tamoxifen N-β-D-Glucuronide	C32H38NO7
Tamoxifen N-Oxide	C26H29NO2

COMPOUND	ELEMENTAL COMPOSITION
Temazepam	C16H13ClN2O2
Temazepam β-D-Glucuronide	C22H21ClN2O8
Desmethyldiazepam	C15H11ClN2O
Trimethoprim	C14H18N4O3
alfa-hydroxytrimethoprim	C14H18N4O4
Venlafaxine	C17H27NO2
N-Desmethyl Venlafaxine	C16H25NO2
O-Desmethyl Venlafaxine	C16H25NO2
O-Desmethyl Venlafaxine β-D-Glucuronide	C22H33NO8
N,N-Didesmethyl-O-desmethyl Venlafaxine Glucuronide	C20H29NO8
N,O-Didesmethyl Venlafaxine	C15H23NO2
N,N-Didesmethyl-O-desmethyl Venlafaxine	C14H21NO2
N,O-Didesmethyl Venlafaxine β-D-Glucuronide	C21H31NO8

3.2.3 Discusión de los resultados (artículo científico 5)

En trabajos previos realizados en nuestro laboratorio ya se había realizado un *screening* de unos 350 fármacos en aguas de efluente urbano. Para 77 de estos fármacos se disponía del patrón comercial y por lo tanto, se conocía su tiempo de retención, sus iones fragmento y su distribución isotópica. Esta información resultó de gran ayuda para poder detectar e identificar de forma rápida los compuestos.

Como se ha explicado en la Introducción, los espectros de masas se pueden procesar y evaluar cuando se desee utilizando bases de datos nuevas o modificadas. En este trabajo se generó una nueva base de datos de, aproximadamente, 160 metabolitos procedentes de unos 50 fármacos. Dicha base de datos incluía la fórmula molecular y las masas teóricas de los metabolitos. Al no disponer de ningún patrón no conocíamos ningún dato experimental (tiempo de retención y fragmentación).

En primer lugar, se procesaron los espectros de masas obtenidos a baja energía de colisión (4 eV) utilizando el software ChromaLynx XS en modo *target*. Este modo de análisis se basa en la búsqueda de la m/z exacta teórica de los compuestos incluidos en la base de datos. En aquellos casos en los que se observó un pico cromatográfico (extraído con una nw-XIC de 0.02 Da), que podría corresponder a un posible metabolito, se evaluó su error de masa y su *pattern* isotópico.

La segunda parte de este proceso consistió en la identificación tentativa de los metabolitos detectados. Para ello, se estudiaron los iones fragmentos de los espectros a baja y a alta energía (con una rampa de energía de colisión de 15 a 40 eV). Como era de esperar, estos últimos proporcionaron una información mucho más rica pues a alta energía de colisión se favorece la fragmentación de las moléculas.

Para la confirmación de los metabolitos se siguieron dos estrategias distintas:

- Patrón del fármaco disponible en el laboratorio

  Cuando disponíamos del patrón correspondiente al fármaco del que procedían los metabolitos el punto de partida fueron los iones fragmento de dicho fármaco. Conociendo la fragmentación del fármaco y teniendo en cuenta las diferencias estructurales entre éste y el metabolito candidato, se predecía la composición elemental de los posibles iones fragmento y se calculaba su masa exacta. Tras realizar los XICs a las masas exactas esperadas, se comprobaba si el tiempo de retención de los picos obtenidos coincidía con el de la molécula (des)protonada del metabolito. En caso afirmativo, parecía más que razonable pensar que el compuesto detectado se trataba del metabolito sospechoso. Además, estos fragmentos se comparaban con los iones producto reportados en otros trabajos previamente publicados, siempre que esta información estuviera disponible. La confirmación inequívoca se obtendría al comprar e inyectar el patrón de referencia, en el caso de que estuviera disponible comercialmente.

  De este modo se identificaron tres metabolitos: N-desmetil claritromicina, 14-hidroxi claritromicina y un metabolito del omeprazol, pero tan sólo se pudo adquirir el patrón de referencia del primer compuesto. El metabolito 14-hidroxi claritromicina no estaba disponible comercialmente. La detección e identificación del metabolito del omeprazol fue el caso más complicado. Nuestra base de datos contenía tres metabolitos del omeprazol isómeros (m/z 316.1120). Al realizar un XIC en el espectro a baja energía de colisión a dicha masa se obtuvieron dos picos cromatográficos (5.55 y 6.03 min). Para dos de los posibles metabolitos (4-desmetoxi omeprazol y 4-hidroxi omeprazol sulfuro), la composición elemental del principal ion fragmento esperado era la misma (m/z 168.0483). Al realizar un XIC a alta energía de colisión a dicha masa se obtuvo un pico cromatográfico a 6.04 min. En cambio, al realizar un XIC a alta energía a la masa esperada para el otro metabolito candidato (5-O-desmetil omeprazol sufuro, m/z 182.0640) no se obtuvo ningún pico, por lo que se descartó que los picos

cromatográficos observados (5.55 y 6.03 min) correspondieran a su presencia. Para tratar de identificar cuál de los dos posibles candidatos correspondía al compuesto que eluía a 6.03-6.04 minutos se realizaron diferentes XICs de sus otros posibles fragmentos (m/z 136.0762 para 4-hidroxi omeprazol sulfuro y 120.081 para 4-desmetoxi omeprazol) pero no se obtuvo ningún pico cromatográfico debido probablemente a la baja abundancia de este ion. Tan sólo la adquisición de los dos patrones de referencia habría permitido identificar el metabolito en cuestión. En el momento de realizar este trabajo tan sólo el patrón de 4-hidroxi omeprazol sulfuro estaba disponible comercialmente. Tras su adquisición y posterior inyección se comprobó que el compuesto eluía a 6.03 minutos y que los fragmentos obtenidos a alta energía de colisión coincidían con los que se habían predicho. Sin embargo, al no disponer del patrón de 4-desmetoxi omeprazol, no fue posible descartar con seguridad entre los dos posibles candidatos.

- <u>Patrón del fármaco no disponible en el laboratorio</u>

  En ausencia de información experimental sobre la fragmentación del fármaco de partida, el software *MassFragment* resultó ser una herramienta útil para deducir la estructura de los fragmentos de los posibles metabolitos.

  Tras realizar los XICs de los diferentes iones observados en el espectro a alta energía, se seleccionaron aquellos cuyo tiempo de retención coincidía con el de la molécula (des)protonada. El software empleado propone las estructuras de los iones fragmento y calcula el error de los fragmentos obtenidos experimentalmente respecto a las masas teóricas propuestas. Estos fragmentos se compararon con los iones producto reportados en bibliografía. La adquisición e inyección del patrón de referencia del metabolito candidato fue el paso decisivo para confirmar su identidad.

Siguiendo esta estrategia se identificaron dos metabolitos: el ácido carboxílico de clopidogrel y el ácido fenofíbrico. En ambos casos, los errores de las masas exactas experimentales fueron menores de 2 mDa respecto a las masas teóricas propuestas. Además, el espectro combinado del pico cromatográfico $[M+H]^+$ de ambas moléculas correspondía al *pattern* isotópico típico de una molécula con un átomo de cloro y estaba en concordancia con la composición elemental de las moléculas de los dos metabolitos candidatos. Todos los iones fragmento propuestos para el ácido carboxílico de clopidogrel y la mayoría de los fragmentos del ácido fenofíbrico conservaron este átomo de cloro, en conformidad con el espectro de masas.

En la Figura 3.2 se muestra de manera esquemática las estrategias empleadas en esta Tesis para la identificación de los metabolitos.

La información obtenida de cada uno de los metabolitos identificados (iones fragmento y tiempo de retención) se incorporó posteriormente a nuestra base de datos para facilitar la detección e identificación de estos metabolitos en futuros análisis.

Los cuatro metabolitos detectados cuyo patrón habíamos adquirido comercialmente para confirmar su identidad (N-desmetil claritromicina, ácido fenofíbrico, ácido carboxílico de clopidogrel, y 4-hidroxi omeprazol sulfuro) se incluyeron en un método multirresidual basado en LC-MS/MS (QqQ) con objeto de disponer de un método cuantitativo de análisis en aguas a bajos niveles de concentración. Para más detalle ver *artículo científico 7*.

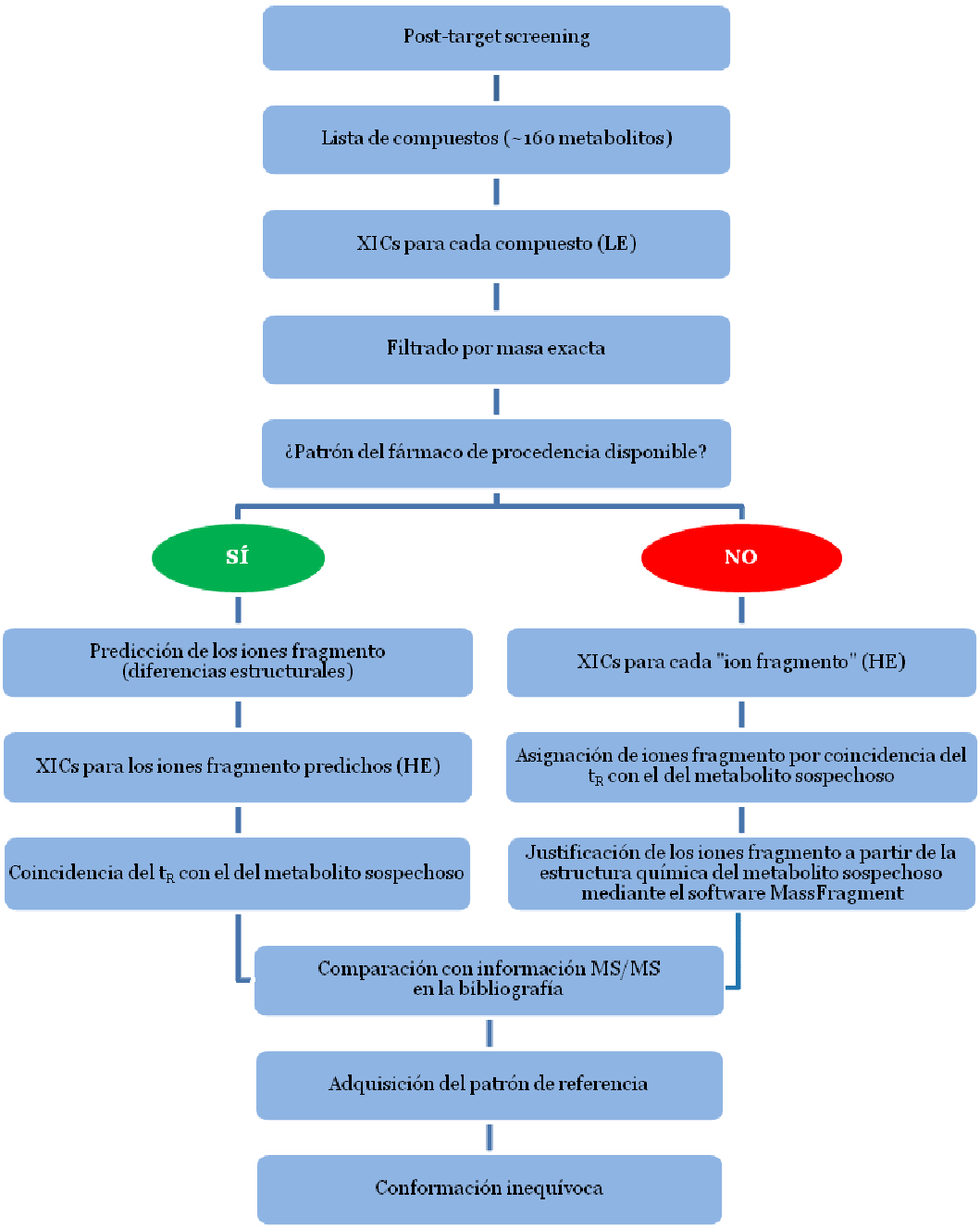

Figura 3.2 Diagrama de flujo de las estrategias utilizadas para la identificación de los metabolitos. (Abreviatura: LE= baja energía de colisión; HE = alta energía de colisión; t_R = tiempo de retención)

3.2.4 Artículo científico 6

Research Article

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Importance of MS selectivity and chromatographic separation in LC-MS/MS-based methods when investigating pharmaceutical metabolites in water. Dipyrone as a case of study

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Pharmaceuticals are emerging contaminants of increasing concern because of their presence in the aquatic environment and potential to reach drinking-water sources. After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form, as the parent compound, and/or as free or conjugated metabolites. Determination of most pharmaceuticals and metabolites in the environment is commonly made by liquid chromatography (LC) coupled to mass spectrometry (MS). LC coupled to tandem MS is the technique of choice nowadays in this field. The acquisition of two selected reaction monitoring (SRM) transitions together with the retention time is the most widely accepted criterion for a safe quantification and confirmation assay. However, scarce attention is normally paid to the selectivity of the selected transitions as well as to the chromatographic separation. In this work, the importance of full spectrum acquisition high-resolution MS data using a hybrid quadrupole time-of-flight analyser and/or a suitable chromatographic separation (to reduce the possibility of co-eluting interferences) is highlighted when investigating pharmaceutical metabolites that share common fragment ions. For this purpose, the analytical challenge associated to the determination of metabolites of the widely used analgesic dipyrone (also known as metamizol) in urban wastewater is discussed. Examples are given on the possibilities of reporting false positives of dypirone metabolites by LC-MS/MS under SRM mode due to a wrong assignment of identity of the compounds detected.

Keywords: dipyrone; pharmaceutical metabolites; ultrahigh pressure liquid chromatography; time-of-flight mass spectrometry; MS^E; urban wastewater; false positives

INTRODUCTION

The presence of pharmaceuticals in the environment is a matter of concern because of their wide consumption and potential negative effect on the water quality and living organisms.[1,2] After human and/or veterinary consumption, pharmaceuticals are excreted in unchanged form, as the parent compound, and also as free and/or conjugates metabolites. Once pharmaceuticals reach the aquatic environment, both the parent compound and metabolites can suffer different transformation processes, mainly photodegradation and hydrolysis, producing a variety of transformation products in water.

Most recent methods for the determination of pharmaceuticals in the environment are based on the use of liquid chromatography coupled to mass spectrometry (LC-MS), and particularly on LC-tandem mass spectrometry (LC-MS/MS) using triple quadrupole (QqQ) analyser.[3,4] The usual approach has been the acquisition of two transitions in LC-MS/MS methods, which fulfills existing guidelines.[5,6] Confirmation of positive findings is based on the accomplishment of ion ratio and retention time, which must fit the limits established by current guideliness. Under these circumstances, not much attention is paid to the chromatographic separation and/or transitions selectivity, assuming that other compounds present in the sample will not fulfil these criteria.

The satisfactory sensitivity in full spectrum acquisition mode, high resolution (HR), exact mass measurements and MS/MS capabilities of hybrid quadrupole time-of-flight (QTOF) mass spectrometry make of this technique a powerful analytical tool for the identification and confirmation of organic contaminants. Moreover, and opposed to analytical methods based on selected ion monitoring or selected reaction monitoring (SRM), accurate-mass full-spectrum data generated by LC-(Q)TOF MS remain available over time. This allows investigating any other compound in addition to target analytes, provided such compound has passed the sample preparation, chromatographic separation and ionization process with sufficient efficiency. This fact clearly represents an important advantage of HRMS for wide scope screening of organic contaminants.[7–9] In the last years, there has been a growing trend on using TOF MS for screening and confirmation of pharmaceuticals in environmental samples.[4,10–12] However, it has been scarcely used to investigate pharmaceutical metabolites in the environment.[13]

Dipyrone (DIP, also known as metamizol) is a non-steroidal anti-inflammatory drug, commonly used to treat severe pain associated with colic, cancer and migranes.[14] It is marketed under various trade names, including *Conmel*®, *Neo-Melubrina*®,

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ABSTRACT

Pharmaceuticals are emerging contaminants of increasing concern because of their presence in the aquatic environment and potential to reach drinking-water sources. After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form, as the parent compound, and/or as free or conjugated metabolites. Determination of most pharmaceuticals and metabolites in the environment is commonly made by liquid chromatography (LC) coupled to mass spectrometry (MS). LC coupled to tandem MS is the technique of choice nowadays in this field. The acquisition of two selected reaction monitoring (SRM) transitions together with the retention time is the most widely accepted criterion for a safe quantification and confirmation assay. However, scarce attention is normally paid to the selectivity of the selected transitions as well as to the chromatographic separation. In this work, the importance of full spectrum acquisition high-resolution MS data using a hybrid quadrupole time-of-flight analyser and/or a suitable chromatographic separation (to reduce the possibility of co-eluting interferences) is highlighted when investigating pharmaceutical metabolites that share common fragment ions. For this purpose, the analytical challenge associated to the determination of metabolites of the widely used analgesic dipyrone (also known as metamizol) in urban wastewater is discussed. Examples are given on the possibilities of reporting false positives of dypirone metabolites by LC-MS/MS under SRM mode due to a wrong assignment of identity of the compounds detected.

Keywords

Dipyrone; Pharmaceutical metabolites; Ultrahigh pressure liquid chromatography; Time-of-flight mass spectrometry; MS^E; Urban wastewater; False positives.

1. Introduction

The presence of pharmaceuticals in the environment is a matter of concern because of their wide consumption and potential negative effect on the water quality and living organisms.[1,2] After human and/or veterinary consumption, pharmaceuticals are excreted in unchanged form, as the parent compound, and also as free and/or conjugates metabolites. Once pharmaceuticals reach the aquatic environment, both the parent compound and metabolites can suffer different transformation processes, mainly photodegradation and hydrolysis, producing a variety of transformation products in water.

Most recent methods for the determination of pharmaceuticals in the environment are based on the use of liquid chromatography coupled to mass spectrometry (LC-MS), and particularly on LC-tandem mass spectrometry (LC-MS/MS) using triple quadrupole (QqQ) analyser.[3,4] The usual approach has been the acquisition of two transitions in LC-MS/MS methods, which fulfills existing guidelines.[5,6] Confirmation of positive findings is based on the accomplishment of ion ratio and retention time, which must fit the limits established by current guideliness. Under these circumstances, not much attention is paid to the chromatographic separation and/or transitions selectivity, assuming that other compounds present in the sample will not fulfil these criteria.

The satisfactory sensitivity in full spectrum acquisition mode, high resolution (HR), exact mass measurements and MS/MS capabilities of hybrid quadrupole time-of-flight (QTOF) mass spectrometry make of this technique a powerful analytical tool for the identification and confirmation of organic contaminants. Moreover, and opposed to analytical methods based on selected ion monitoring or selected reaction monitoring (SRM), accurate-mass full-spectrum data generated by LC-(Q)TOF MS remain available over time. This allows investigating any other compound in addition to target analytes, provided such compound has passed the sample preparation, chromatographic separation and ionization process with sufficient efficiency. This fact clearly represents an important advantage of HRMS for wide scope screening of organic contaminants.[7-9] In the last years, there has been a growing trend on using TOF MS for screening and confirmation of pharmaceuticals in environmental samples.[4, 10-12] However, it has been scarcely used to investigate pharmaceutical metabolites in the environment.[13]

Dipyrone (DIP, also known as metamizol) is a non-steroidal anti-inflammatory drug, commonly used to treat severe pain associated with colic, cancer and migranes.[14] It is marketed under various trade names, including *Conmel®, Neo-Melubrina®, Magnopyrol®, Prodolina F®, Analgin*®, *Nolotil*® and *Novalgin*®. Immediately after its introduction in 1922, the use of DIP was linked to serious toxic effects, such as dose-independent teratogenicity, increased bleeding times and a potentially fatal agranulocytosis.[15] While the use of DIP has been banned in USA, Canada or UK, in many Western European countries (e.g. Germany, Italy or Spain), it is still one of the most consumed pharmaceuticals.[16] DIP is considered a pro-drug. After oral intake, it is rapidly hydrolyzed to 4-methylaminoantipyrine (4-MAA), which is absorbed and bio-transformed by enzymatic reactions. In the liver, 4-MAA is metabolized to 4-aminoantipyrine (4-AA) via demethylation and further acetylated to 4-acetylaminoantipyrine (4-AAA). Another metabolite, 4-formylaminoantipyrine (4-FAA), is generated by oxidation of the methyl group.[16] The presence of these DIP metabolites has been reported in sewage treatment plant effluents [12, 17] and in surface water.[17]

In a previous work,[18] the presence of 4-amino antipyrine (4-AA) in urban wastewater was reported based on the monitoring of two SRM transitions using a LC-MS/MS method. A slight shift in retention time together with the presence of clear shoulders in the chromatographic peak was observed. However, both retention time and ion ratio deviations as regards the reference standard were within the tolerances established. Despite all requirements established by current guidelines were satisfied, reasonable doubts about a false positive due to unresolved interferences might be hypothesized. The goal of this paper is to point out the benefits of using full spectrum acquisition HRMS, and the need of applying efficient chromatographic separation in some particular complex situations, as for example when investigating pharmaceutical metabolites. For this purpose, the analytical challenge associated to the determination of DIP metabolites in urban wastewater is studied in depth making use of QTOF and QqQ analysers both coupled to UPLC.

2. Experimental

2.1. Reagents and chemicals

Reference standards (purity higher than 93%) of 4-AA, 4-AAA and 4-FAA were purchased from Sigma–Aldrich (St Louis, MO, USA) and Toronto Research Chemicals (Ontario, Canada).

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), Sodium hydroxide >99% (NaOH), ammonia solution (25%), and formic acid (98–100%) were acquired from Scharlau (Barcelona, Spain). Leucine encephalin (used as the lock mass) was purchased from Sigma-Aldrich.

2.2. Instrumentation

UHPLC-QTOF MS

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI interface operating in positive ion mode. The chromatographic separation was performed using an Acquity UPLC BEH C18 1.7-µm particle size analytical column 100 × 2.1 mm (Waters) at a flow rate of 300 µL/min. The mobile phases used were A = H_2O with 0.01% HCOOH and B = MeOH with 0.01% HCOOH. The initial percentage of B was 10%, which was linearly increased to 90% in 14 min, followed by a 2-min isocratic period and, then, returned to initial conditions during 2 min in total run duration of 18 min. Nitrogen was used as the drying gas and nebulizing gas. The gas flow was set at 1200 L/h. TOF-MS resolution was approximately 25 000 at full width half maximum at *m/z* 556. MS data were acquired over an *m/z* range of 50–1200. A capillary voltage of 0.7 kV and cone voltage of 20 V were used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 600℃ and the sour ce temperature to 120℃. The column temperature was set to 40℃.

For MS^E experiments, two acquisition functions with different collision energies were created. The low energy function (LE), selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV in order to obtain a greater range of fragment ions. The LE and HE functions settings were for both a scan time of 0.3 s.

Calibrations were automatically conducted from *m/z* 50 to 1200 with a 1:1 mixture of 0.05-M NaOH:5% HCOOH diluted (1:25) with acetonitrile:water (80:20). For automated accurate mass measurement, the lock-spray probe was used, using as lockmass a solution of leucine enkephalin (2 µg/mL) in acetonitrile:water (50:50) at 0.1% HCOOH pumped at 20 µL/min. *m/z* 556.2771 and *m/z* 278.1141 (corresponding to the protonated molecule of leucine enkephalin

and to a fragment ion, respectively) were used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time.

UPLC-MS/MS

For MS/MS experiments on the triple quadrupole (TQD) mass spectrometer, chromatographic separation was performed using an Acquity UPLC HSS T3 column (C_{18}), 1.8 µm, 100 × 2.1 mm (Waters) at a flow rate of 300 µL/min. Mobile phase consisted of a water/methanol, both 0.1-mM NH_4Ac and 0.01% HCOOH, gradient. The methanol percentage was changed linearly as follows: 0 min, 5%; 5 min, 90%; 6 min, 90%; 6.1 min; 5%. Analysis run time was 10 min. The sample injection volume was 20 µL. A TQD mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters) was used. Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N2 LC–MS (Claind, Teknokroma, Barcelona, Spain). The cone gas and the desolvation gas flows were set at 60 L/h and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a performance of $2 \times 10 - 3$ mbar in the T-Wave collision cell. A capillary voltage of 3.5 kV (positive ionization mode) and a cone voltage of 20 V were applied. The interface temperature was set to 500℃, and the sou rce temperature to 120℃. A dwell time of 0.01 s was selected. Selected SRM transitions were the following: 204.4 > 56.1 and 204.4 > 83.1 for 4-AA, 232.4 > 56.1 and 232.4 > 214.3 for 4-FAA and, 246.4 > 83.1 and 246.4 > 228.3 for 4-AAA.

2.3. Water samples

Twenty-four effluent wastewaters extracts from the Spanish Mediterranean area, previously analysed by UHPLC-QqQ MS after being subjected to solid-phase extraction with Oasis HLB (60 mg),[18] were re-injected by UHPLC-(Q)TOF MS in order to investigate the possible presence of 4-AA and other metabolites of DIP.

3. Results and discussion

In a previous work, we developed a target method[18] based on UHPLC-MS/MS with TQD that was applied to the determination of 45 pharmaceuticals in water. As scientific literature shows, pharmaceutical metabolites have hardly been studied in environmental water and wastewater. Typically, only metabolites of those compounds classified as pro-drugs have been included in target methods.[19-21] This is the case of 4-AA (the main reported metabolite of DIP) and salicylic acid (the main metabolite of acetylsalicylic acid) that were also included in our previous work.[18] 4-AA was found in around 80% of the effluent wastewater samples analysed, with a maximum concentration of 2.8 µg/L. The two transitions selected, one for quantification (Q) 204 > 56, and the other for confirmation (q) 204 > 83, were observed in the samples and Q/q ratios were within the maximum deviations admitted. Under the chromatographic conditions employed, a poor chromatographic peak shape was observed in most of samples, presenting a clear shoulder, although the criteria used for confirmation of positives were accomplished. Therefore, the presence of 4-AA was assumed in the samples.

However, the shoulder observed in the LC-MS/MS chromatograms for the two transitions monitored encouraged us to investigate the presence of this metabolite in more detail to discard a false positive. The same water samples extracts were subsequently analysed by UHPLC-(Q)TOF MS under MS^E mode. With MS^E experiments, both (de)protonated molecule and fragment ion data are enabled in a single acquisition, without the need of selecting the precursor ion, a notable difference with true MS/MS experiments.[8, 22, 23]

Figure 1 shows LE and HE spectra of the 4-AA reference standard. The elemental composition for up to seven main fragments observed in the HE TOF MS spectrum was calculated, obtaining errors for accurate masses normally below 0.5 mDa in relation to the theoretical predicted exact masses. The structures suggested by MassFragment software are also depicted, showing that the product ions previously selected for SRM transitions (*m/z* 56 and 83) seem selective enough.

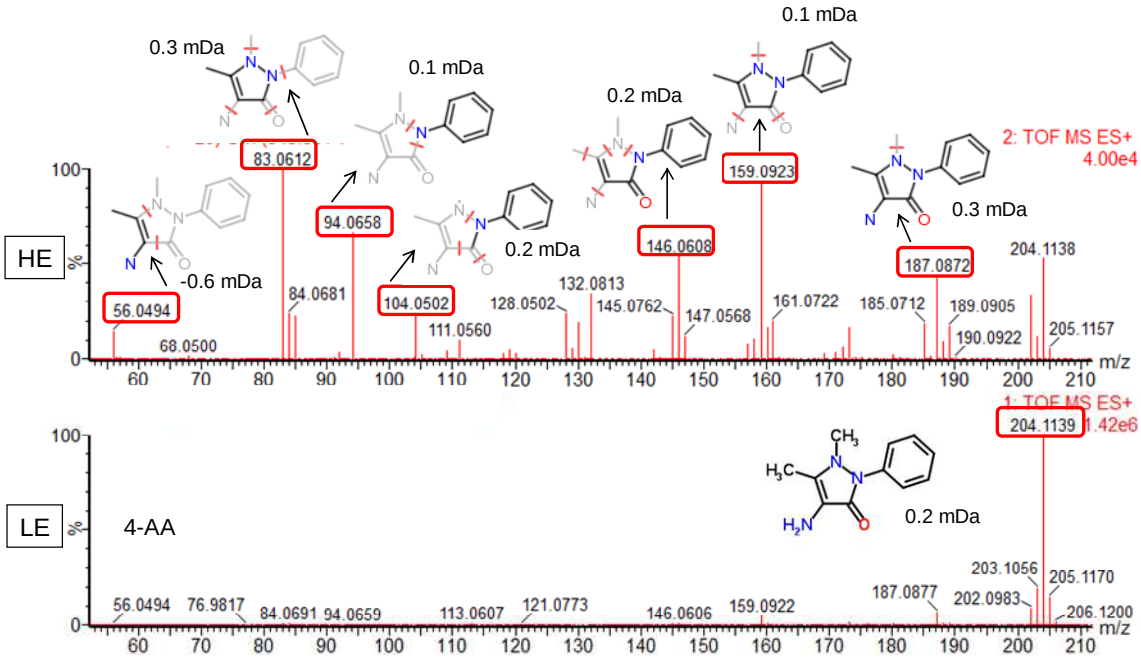

Figure 1. LE and HE spectra of 4-aminoantipyrine (4-AA). Structures proposed by MassFragment software for fragment ions of this compound.

In the samples analysed by QTOF MS, the narrow-window extracted ion chromatograms (nw-XIC) (20-mDa mass window)[24, 25] at the exact mass of 4-AA protonated molecule (*m/z* $[M + H]^+$ 204.1137) were obtained from LE MS data. In several samples, two close peaks were observed, as shown in Fig. 2a. The chromatographic peak at 3.33 min was assigned, in principle, to 4-AA as the accurate mass (*m/z* 204.1132, Fig. 2b) differed −0.5 mDa from the theoretical mass of 4-AA, and its retention time did not exceed the maximum deviation of 2.5% typically allowed in LC-MS (reference retention time, 3.25 min). However, the accurate mass of the second peak, at 3.45 min (*m/z* 204.1142, Fig. 2c) also differed only 0.5 mDa from the theoretical mass of 4-AA. When performing the nw-XICs at the expected exact masses of 4-AA fragment ions (HE function), two chromatographic peaks were observed in each case at *m/z* 159, 146, 104, 83 and 56, at both retention times (Fig. 2a). This fact strongly supported that the identity of the compound at 3.33 min was 4-AA. Obviously, the other peak at 3.45 min should correspond to a highly related compound.

However, the mass spectra of the compound eluting at 3.33 min presented two differentiated ions in the LE spectrum, at *m/z* values higher than the 4-AA protonated molecule. One of those was a highly abundant ion at *m/z* 232.1088 (Fig. 2b), and the other was a fragment ion at *m/z* 214.0982. The later was more visible in the HE spectrum (*m/z* 214.0976). The nw-XICs at *m/z* 232.1088 for the LE and *m/z* 214.0976 for the HE function showed the presence of peaks at the same retention time with the same peak shape (Fig. 2a). Thus, it seemed that the accurate mass of the protonated molecule for this compound was 232.1088, and not 204.1132 as initially believed. In addition, the *m/z* 214.0976 ion could be explained as a fragment derived from 232 due to a water loss. Obviously, the compound detected was not 4-AA, but it should be related with it as both shared up to six fragment ions. After searching in available database,[13] this compound was identified as 4-FAA.

In a subsequent step, the reference standard was acquired and injected, confirming the identity of this compound. Hence, the presence of 4-AA was wrongly assigned when this sample was analysed by LC-MS/MS QqQ using the transitions 204 > 56 (Q) and 204 > 83 (q). It seems that 4-FAA produced an in-source fragment at *m/z* 204, which was afterwards isolated and fragmented in the collision cell to give the product ions at *m/z* 56 and 83. In addition, both compounds eluted in our chromatographic system at nearly the same retention time, making likely the reporting of 4-AA false positives. The occurrence of 4-FAA in wastewater has been previously investigated by Martínez-Bueno *et al.*[12] This metabolite was detected in 14 out of the 19 samples analysed, at concentrations between 0.04 and 10 µg/L.

Regarding the peak at 3.45 min, the LE spectrum presented an abundant ion at *m/z* 246.1252 and the HE spectra at *m/z* 228.1137 (also corresponding to a water loss) (Fig. 2c). The nw-XICs at these masses showed the presence of a chromatographic peak at the same retention time (3.45 min) (Fig. 2a). Again, it seemed that this compound was closely related to 4-AA, as both shared up to six fragment ions. After searching in available database,[13] this compound was identified as 4-AAA. As before, the reference standard was subsequently acquired and injected, confirming the identity of the compound. This metabolite has been also reported in wastewater at concentrations between 2 and 25 µg/L.[12]

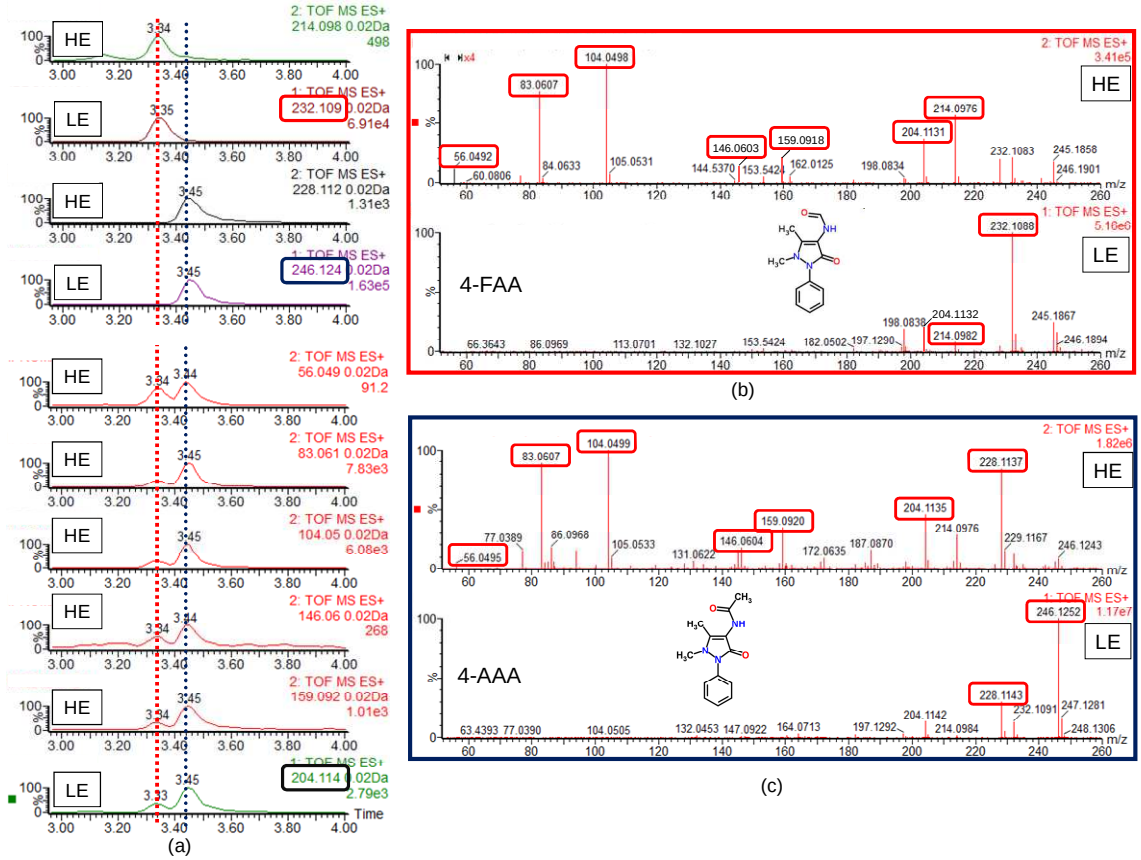

Figure 2. Detection and identification of 4-formylaminoantipyrine (4-FAA) and 4-acetylaminoantipyrine (4-AAA) in an effluent wastewater by UHPLC-QTOF under MS^E mode: (a) XICs (20-mDa mass window) for $[M + H]^+$ in LE function and for the main fragments in HE function; (b) LE and HE spectra of 4-FAA in the sample (chromatographic peak at 3.33 min); (c) LE and HE spectra of 4-AAA in the sample (chromatographic peak at 3.45 min).

Thus, in this particular water sample, the metabolite detected was not 4-AA, but 4-AAA and 4-FAA. A false positive of 4-AA would have been reported if they were not chromatographically resolved. Under the chromatographic conditions employed in the UPLC-QTOF MS, the three metabolites were roughly resolved as all eluted at close retention times. As illustrative example, Fig. 3 shows the UPLC-TOF MS chromatograms for a wastewater sample that was positive for the three metabolites, and where the presence of 4-AA could be unambiguously reported preventing its false identification. However, if no efficient

chromatographic separation occurs for these metabolites, the presence of 4-AA cannot be ensured, even using HR in MS/MS mode, as derived from Müller *et al.*[26] In that paper, 4-AA and 4-AAA perfectly co-eluted at 6.0 min. As 4-AA shares all its product ions with 4-AAA, the detection of the ion at *m/z* 204 might be related with an in-source fragment of 4-AAA and not with the presence of 4-AA. Therefore, to prevent such possibility, it is crucial an improvement in the chromatographic separation for minimising false identifications.

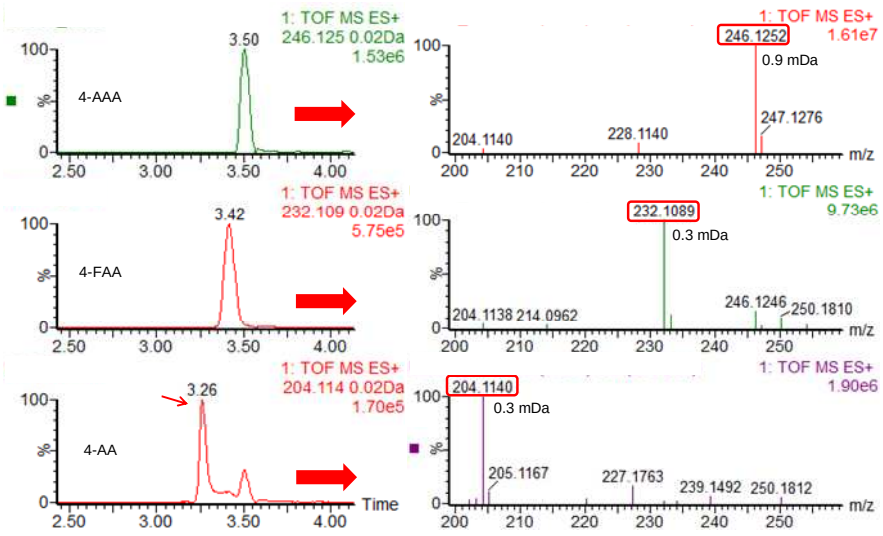

Figure 3. UHPLC-QTOF MS XICs chromatograms and LE spectra for a wastewater sample containing 4-AA, 4-FAA and 4-AAA.

On the basis of these results, the sample extracts previously analysed were injected again in the LC-MS/MS system, with a slight modification in the gradient slope and also considering the transitions for the two additional metabolites identified by TOF MS. Regarding the transitions selected for 4-AA, the cone voltage was reduced down to 20 V, to minimise in-source fragmentation of the other metabolites. Figure 4 shows UHPLC-MS/MS chromatograms for a reference standard containing 4-AA, 4-AAA and 4-FAA, as well as for one of the positive wastewater samples reanalysed. As it can be seen, 4-AA eluted last, opposite to the results obtained when working with QTOF MS, where this compound eluted first. Additional experiments were performed in order to clarify if the change in elution order was due to the different column or to the different mobile phase additives. Both columns (HSS T3 and BEH

C18) were tested using only 0.01% formic acid, or 0.01% formic acid plus 0.1-mM NH_4Ac in the mobile phase. As shown in Fig. 5, the presence of 0.1-mM NH_4Ac in the mobile phase seemed to be the responsible of this change. It is interesting to remark the higher retention of the analytes in the HSS T3 column (based on a C_{18} high strength silica stationary phase) than in the BEH C18 (based on ethylene bridged hybrid technology).

In the particular example shown in Fig. 4B, the presence of 4-AA was in principle thought after being detected in the initial LC-MS/MS analysis. However, when this sample was re-analysed under the new conditions, only small traces of 4-AA were detected, well resolved from the in-source fragments produced by the presence of high concentrations of 4-AAA and 4-FAA.

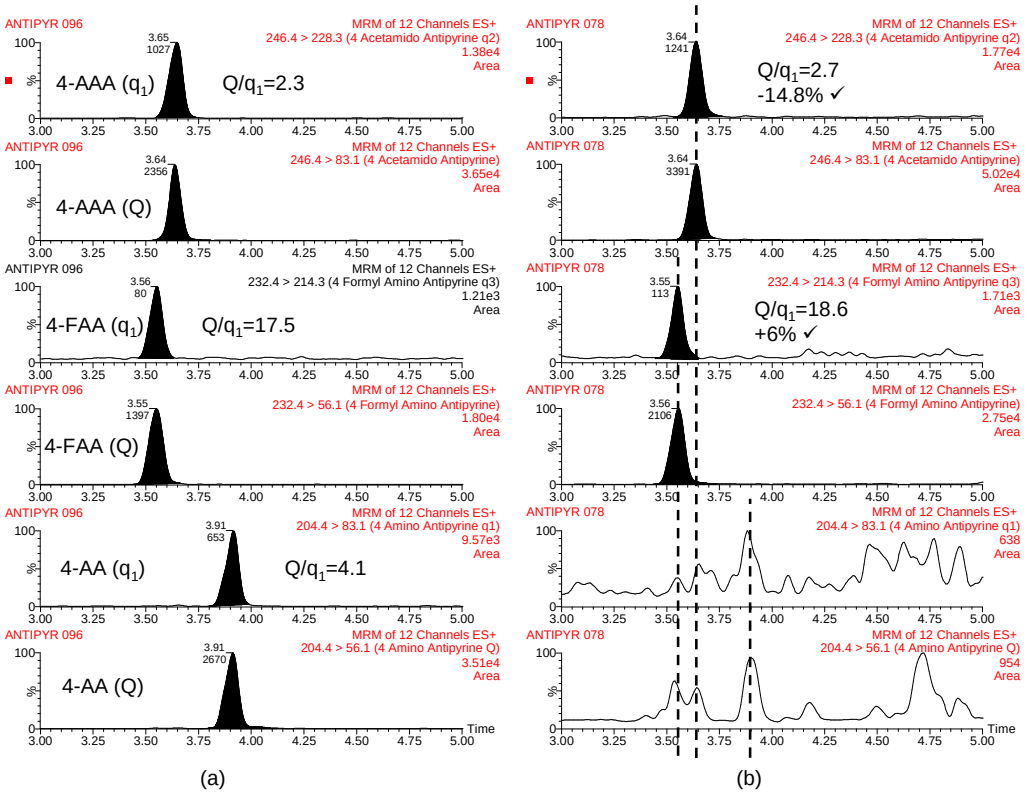

Figure 4. UHPLC-MS/MS (QqQ) chromatograms of 4-AA, 4-FAA and 4-AAA obtained for (a) 25-ng/mL reference standard and (b) wastewater sample containing 4-FAA and 4-AAA, after optimized chromatographic conditions.

After re-analysing the water samples using different chromatographic conditions and selecting specific transitions for the two additional metabolites, it was feasible to discover the presence of metabolites other than 4-AA, initially selected as the only target analyte for DIP. 4-AA was now detected in only 36% of samples analysed, whereas both 4-AAA and 4-FAA were found in around 90% of the samples.

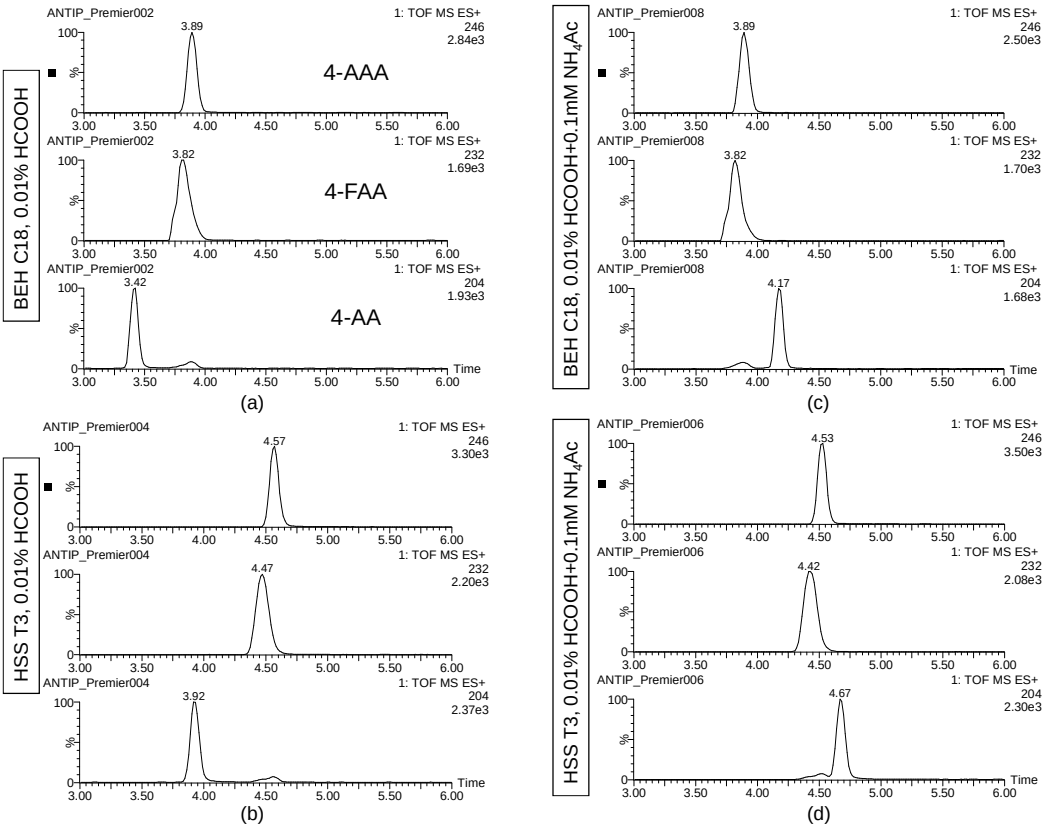

Figure 5. UHPLC-QTOF MS XICs chromatograms for a reference standard containing 4-AA, 4-FAA and 4-AAA (a) BEH C18 column, using only 0.01% formic acid in the mobile phase; (b) HSS T3 column, using only 0.01% formic acid in the mobile phase; (c) BEH C18, using both 0.01% formic acid and 0.1-mM NH_4Ac in the mobile phase. (d) HSS T3 column, using both 0.01% formic acid and 0.1-mM NH_4Ac in the mobile phase.

The examples shown illustrate the importance of using full spectrum acquisition HRMS as well as suitable chromatographic separation to reduce the possibility of bi-isobaric co-eluting 'interferences' when investigating metabolites. These interferences might be due to unexpected metabolites that share in-source fragment ions. Surely, the problems of false identifications might have been ignored if different chromatographic conditions would have been applied, and/or if the three metabolites would have been selected as target analytes in initial analysis. However, we would like to emphasize that false positives might occur in particular situations like that described in this article, where metabolites of pharmaceuticals, and surely of many other organic contaminants like pesticides, drugs of abuse, etc. are determined even using a powerful selective technique as LC-MS/MS under SRM mode.

4. Conclusions

In this work, we illustrate the problems that may occur when investigating the presence of the pro-drug DIP, selecting the metabolite 4-AA as biomarker in the LC-MS/MS analysis of environmental water. The use of HRMS helped to discover that other metabolites of DIP (e.g. 4-FAA and 4-AAA) produced 4-AA via in-source fragmentation, and consequently shared the same transitions. Under these circumstances, the chromatographic separation was crucial for avoiding the assignment of erroneous identifications. This situation might also occur with other contaminants when investigating metabolites or degradation products. The analyst has to take into account that metabolites of a given compound may share the same fragment ions complicating the right identification of the compound actually present in water.

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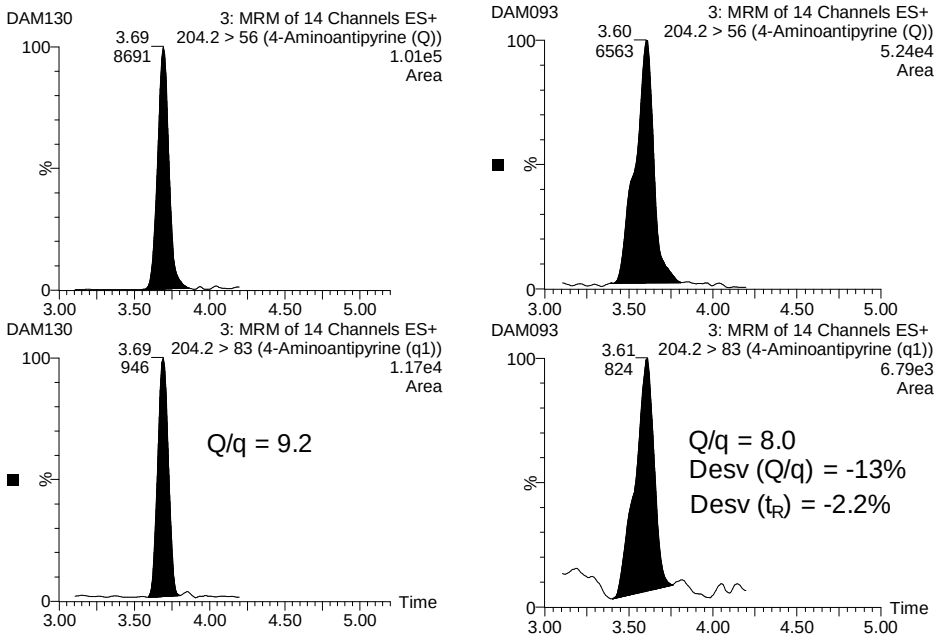
3.2.5 Discusión de los resultados (artículo científico 6)

Como se ha indicado anteriormente, este estudio surgió tras detectar una situación problemática en análisis realizados en aguas mediante LC-MS/MS con triple cuadrupolo. Las transiciones escogidas para la determinación del metabolito de la dipirona, 4-aminoantipirina (4-AA) fueron 204.2 > 56 y 204.2 > 83. En la Figura 3.3 se muestran los cromatogramas de un patrón y de una muestra de efluente. Como se observa, a pesar de que los picos correspondientes a la muestra presentan un hombro, la relación de intensidad entre las transiciones y el tiempo de retención cumplen las tolerancias establecidas.



(a) Patrón **(b)** Muestra de efluente

Figura 3.3 Cromatogramas UHPLC-MS/MS para(a) un patrón (25 µg/L) de 4-aminoantipirina y (b) una muestra de efluente positiva a 4-AA.

Con el fin de investigar la posible presencia del compuesto 4-aminoantipirina (4-AA) y de otros metabolitos de la dipirona, los extractos de muestras de efluente previamente inyectados en el QqQ se reanalizaron mediante LC-QTOF en modo MS^E.

En primer lugar, se inyectó el patrón de referencia de 4-AA. Mediante el uso del software MassFragment se obtuvo la estructura de hasta siete fragmentos (*Figura 1, artículo científico 6*).

En las muestras analizadas por QTOF, el cromatograma generado a partir de la extracción del ion de la molécula protonada de 4-AA (cromatograma XIC, a la masa exacta m/z 204.114) mostraba dos picos no bien resueltos. Uno de ellos aparecía a 3.33 min y el otro a 3.45 min. Al realizar los XIC en el espectro a alta energía (HE, función 2) a las masas de los iones fragmento correspondientes a 4-AA, de nuevo se observaron los dos picos.

Al observar los espectros a baja energía correspondientes a los dos picos cromatográficos nos llamó la atención que, además del ion correspondiente a la molécula protonada de 4-AA ($[M+H]^+$ 204.114), en cada espectro aparecía otro ion con mayor valor de m/z y mayor abundancia. Al realizar un XIC a ambas masas se obtuvieron de nuevo dos picos a 3.34 y 3.45 minutos. Estos iones con mayor valor de m/z y mayor abundancia correspondían a otros dos metabolitos de la dipirona: 4-formilaminoantipirina (4-FAA, m/z $[M+H]^+$ 232.1088, a 3.34 min) y 4-acetilaminoantipirina (4-AAA, m/z $[M+H]^+$ 246.1252, a 3.45 min). Además, junto al ion correspondiente al 4-FAA aparecía un ion fragmento a m/z 214.0976, correspondiente a una pérdida de agua. Análogamente, el espectro de masas correspondiente al pico a 3.45 presentaba, además del ion de 4-AAA, otro correspondiente a una pérdida de agua.

Una vez adquiridos los patrones de referencia la presencia de estos dos metabolitos en la muestra de agua se confirmó tras la inyección de ambos patrones; se obtuvieron sus iones fragmento principales, que resultaron ser los mismos que los del 4-AA.

Por tanto, parece deducirse, a la vista de los espectros de masas, que la muestra de efluente analizada no contenía 4-AA sino los otros metabolitos identificados. La explicación más razonable es que 4-AAA y 4-FAA sufren una fragmentación en la interfase electrospray generando un ion a m/z 204, que corresponde precisamente al 4-AA. Al aislar este ion en la celda de colisión, se fragmenta para dar también los iones producto m/z 56 y 83. En consecuencia, los tres metabolitos comparten las mismas transiciones. Por tanto, si la separación cromatográfica no es suficiente (como la empleada en el método QqQ) la identificación de los metabolitos puede resultar problemática y tanto el 4-FAA como 4-AAA se podrían confundir con el 4-AA. En tal caso, la adquisición de las transiciones SRM correspondientes al 4-AA (en el caso de utilizar un triple cuadrupolo) o incluso la detección del ion m/z 204.114 utilizando espectrometría de masas de alta resolución no permitiría asegurar la presencia de 4-AA. Por lo tanto, es necesario optimizar las condiciones cromatográficas para reducir la posibilidad de coelución de estos compuestos.

Finalmente, los extractos de las muestras se reanalizaron por LC-MS/MS (QqQ) utilizando las condiciones cromatográficas optimizadas e incluyendo las transiciones de los dos metabolitos de la dipirona identificados mediante TOF MS. El metabolito 4-AA se detectó en tan sólo el 36% de las muestras analizadas mientras que 4-FAA y 4-AAA se detectaron en aproximadamente el 90% de las muestras.

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3.3 Determinación de metabolitos seleccionados en aguas medioambientales y residuales mediante UHPLC-MS/MS con analizador de triple cuadrupolo

3.3.1 Introducción

A pesar de que los niveles de concentración de los metabolitos de fármacos detectados en el medio acuático se suelen consideran bajos, de modo que no cabe esperar que provoquen efectos tóxicos, actualmente se desconocen los riesgos que podrían originar a largo plazo. Se necesita disponer de mayor información sobre su presencia y ecotoxicidad para poder conocer el posible impacto de estos compuestos en el medio ambiente. Para ello, saber cuáles son sus niveles de concentración en las aguas resulta fundamental.

La tendencia actual de los métodos multirresiduales es analizar cada vez un mayor número de compuestos. Sin embargo, generalmente se incluyen únicamente los fármacos originales en el listado de analitos, siendo muy pocos los metabolitos que se determinan con estos métodos de análisis. Como se ha apuntado en la Introducción de este capítulo, hasta hace pocos años el número de metabolitos que se analizaba era muy limitado y en la mayoría de las ocasiones tan sólo se consideraban los metabolitos de fármacos clasificados como profármacos. Actualmente, al haber un mayor conocimiento sobre el metabolismo de fármacos, ha aumentado el número de metabolitos que se incluyen en los métodos analíticos. Por ejemplo, Tarcomnicu y col. desarrollaron en el año 2011 un método para el análisis para quince fármacos y cuatro metabolitos utilizando LC-MS/MS con triple cuadrupolo (Tarcomnicu, 2011). Recientemente, se ha publicado un método para la determinación de ochenta fármacos, incluidos ocho metabolitos, haciendo uso de un analizador híbrido cuadrupolo-trampa lineal de iones (QTRAP o QLIT) (Gros, 2012) y otro para diecinueve metabolitos/TPs y otros tantos fármacos mediante preconcentración *on-line* y determinación con triple cuadrupolo (López-Serna, 2012). A pesar de estos recientes avances, su inclusión en las metodologías analíticas sigue siendo muy limitada.

Se podrían señalar diversas causas para justificar la escasez de metabolitos en los métodos multirresiduales. Uno de los principales motivos se encuentra en las dificultades analíticas que conlleva su análisis. Los metabolitos suelen ser compuestos más polares que los fármacos de los que proceden y, en consecuencia, se retienen

menos en los cartuchos de extracción y en la columna de separación cromatográfica en fase reversa. Este hecho provoca que, en ocasiones, no se consiga una buena retención en la fase de preconcentración, o una buena separación entre los analitos o entre éstos y los componentes polares de la matriz que coeluyen, los cuales podrían interferir en la ionización y detección de los metabolitos. Este problema se agrava cuando se analizan matrices complejas, por ejemplo, muestras de agua residual. Además, hay que tener presente que los metabolitos generalmente se encuentran a niveles de concentración muy bajos, por lo que las posibilidades de error aumentan considerablemente.

Otra de los problemas analíticos es la falta de disponibilidad comercial de patrones analíticos, los cuales son necesarios para la cuantificación de los analitos en las muestras.

Por otro lado, nos encontramos con dificultades relacionadas con la falta de información sobre estos compuestos. Aunque en la actualidad antes de aprobar el uso de un fármaco se ha de estudiar su farmacocinética (guía EMEA), existe todavía un cierto desconocimiento sobre el metabolismo y la ecotoxicidad de muchos de ellos ya que se introdujeron en el mercado antes de la aplicación de esta guía. Además, la información que existe sobre metabolismo no siempre está a disposición de la comunidad científica, pues muchas veces forma parte de dosieres del fabricante protegidos por un proceso de confidencialidad hasta vencimiento de la patente. Debido a esta falta de información muchos metabolitos que podrían ser relevantes desde el punto de vista ambiental no se han identificado todavía.

En el artículo que se presenta a continuación se seleccionaron veintiún compuestos, de los cuales catorce eran metabolitos. La selección se basó en varios criterios. En primer lugar, se seleccionaron cuatro metabolitos identificados mediante un análisis retrospectivo de muestras de efluente (*artículo científico 5*) así como los metabolitos de la dipirona identificados en un trabajo previo (*artículo científico 6*). En ambos trabajos se había utilizado un analizador QTOF, cuya aplicación en el análisis cuantitativo es limitada. Por ello, se decidió desarrollar un método de análisis utilizando un analizador de triple cuadrupolo que permitiera cuantificar y confirmar los

compuestos simultáneamente a muy bajos niveles de concentración. Esta lista se completó con otros metabolitos seleccionados en función de su detección en las aguas, según la información encontrada en la literatura científica (Miao, 2003; Kasprzyk-Hordern, 2007; Tarcomnicu, 2011) y de su disponibilidad comercial. Además, se incluyeron los fármacos originales para cada metabolito excepto el clofibrato, fenofibrato y dipirona por tratarse de profármacos.

Siguiendo la metodología general de trabajo propuesta en el *artículo científico 4*, el método desarrollado se validó en seis muestras superficiales y en seis de efluente (un número de muestras muy superior al habitual), con el objetivo de comprobar la robustez del método y su aplicación realista a muestras de diferente origen y composición. En el caso de efluente urbano, fue preciso diluir las muestras para minimizar el efecto matriz, debido a la complejidad de estas muestras y a la escasez de patrones marcados isotópicamente con los que poder corregir el efecto matriz.

3.3.2 Artículo científico 7

The interest of monitoring pharmaceutical metabolites in the aquatic environment

(Enviado para su publicación)

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ABSTRACT

Pharmaceuticals, once ingested, are commonly metabolized in the body into more polar and soluble forms. These compounds might not be completely removed in the wastewater treatment plants and consequently being discharged into the aquatic ecosystem. In this work, a multi-class sensitive method for the analysis of 21 compounds, including 7 widely consumed pharmaceuticals and 14 relevant metabolites, has been developed based on the use of UHPLC-MS/MS in selected reaction monitoring (SRM) mode. The method was validated in a high number of samples: six surface waters (SW) and six effluent wastewaters (EWW) at realistic concentration levels that can be found in waters. The optimized method was applied to the analysis of different types of water samples (rivers, lakes and effluent wastewater), detecting nearly all the parent compounds and metabolites investigated in this work. This fact illustrates that not only pharmaceuticals but also their metabolites are commonly present in waters. Analytical research and monitoring programs should be directed not only towards parent pharmaceuticals but also towards relevant metabolites to have a realistic overview of the impact of pharmaceuticals in the aquatic environment.

Keywords

Pharmaceuticals; Metabolites; Ultra-high performance liquid chromatography; tandem mass spectrometry; Multi-class method; Surface water; Wastewater

1. Introduction

In the last years, many papers dealing with the presence of pharmaceuticals in the aquatic environment have been reported. Most of the work performed until now has been focused on parent pharmaceuticals, while metabolites have been much less investigated. After human and/or veterinary consumption, pharmaceuticals can be excreted in unchanged form as the parent compound and/or as free or conjugated metabolites through urine and/or faeces. Metabolism occurs in two phases. The first one involves typically oxidation, reduction, or hydrolysis, and the second phase consists of transferring a polar group to the parent compound or the metabolite to render a conjugate[1,2]. Obviously, not all pharmaceuticals are metabolised to the same extent. They may be classified in four classes according to the proportions of excreted parent compound[3], i.e. low excretion (≤ 5%), moderately low (6-39%), relatively high (40-69%), and high excretion compounds (≥ 70%). Among the first group, there are some compounds known as pro-drugs, i.e., inactive substances that after their ingestion are converted to an active form in the body.

Both parent pharmaceuticals and metabolites might not be fully eliminated during the treatment processes in wastewater treatment plants (WWTP) being discharged into the aquatic ecosystems through treated wastewaters. Research is commonly focused on parent compounds, and little is known about the presence of metabolites and on transformation products (TPs) that can be formed during water treatment. In fact, only a few works have reported values of pharmaceuticals metabolites and TPs in the aquatic environment[4-7]. Although pharmaceuticals and metabolites are typically found at low concentration levels, the effects derived from the exposure to a mixture of parent pharmaceuticals and their metabolites are still largely unknown. Moreover, some of the metabolites are still bioactive and may have high stability and mobility in the environment[8]. Mompelat et al.[1] have recently reported that only around 30 pharmaceutical by-products (including metabolites and transformation products) have been included in environmental investigations.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with triple quadrupole triple quadrupole (QqQ) analyzer is nowadays the technique of choice for trace analysis of pharmaceuticals due to the high selectivity and sensitivity achieved in selected reaction monitoring (SRM) mode. LC-MS/MS has been commonly used for multi-class determination of pharmaceuticals compounds, normally including only a few metabolites in the target list of analytes[9-11]. In the last two years, this trend is being changing, as more metabolites

are included in the analytical methods. For example, Tarcomnicu et al.[11] have developed a method for the analysis of different pharmaceuticals and four metabolites. Recently, a method based on automated off-line solid phase extraction (SPE) using a triple quadruple-linear ion trap mass spectrometer (QqLIT) has allowed the determination of 8 metabolites[8]. Another LC-MS/MS method has been reported using QqQ for the analysis of 19 pharmaceutical metabolites and TPs[12]. In addition, a few papers have been published on investigation of pharmaceutical metabolites and TPs by high-resolution mass spectrometry (HR MS), emphasizing the use of hybrid quadrupole time-of-flight (QTOF)[13-16]. LC-HR MS has been proven to be a powerful and promising approach to investigate these compounds in waters from reported-known metabolites/TPs to unknown compounds that share common fragments with the parent molecule[13,17].

Some works have reported metabolite concentrations higher than the original molecule[7,18]. This also supports the interest of searching for metabolites to have a wider and more realistic knowledge about the impact of pharmaceuticals in the aquatic environment. To this aim, multi-class methods including pharmaceuticals and metabolites are required, but this type of analysis presents some difficulties, as metabolites are usually more polar than parent compounds. This makes problematic their simultaneous extraction and LC determination, as they are less retained on the SPE cartridges and on the commonly used reversed-phase LC columns. In addition, the low concentrations normally present in waters require the use of highly sensitive methods for their determination. This is especially important in complex environmental matrices where the presence of co-extracted sample matrix components results in ionization suppression or enhancement effects. Although matrix effects can be corrected using isotope-labelled internal standards (ILIS)[19], the availability of ILIS reference standards is rather limited in comparison with parent pharmaceuticals. And last but not least, it is necessary to ensure the confident identification of the compound detected. This issue might be problematic for isomeric metabolites that share common fragments with the parent compound, or metabolites that can generate the parent compound as an in-source fragments in the LC-MS instrument[17]. Under this situation, is necessary to maximize precautions to ensure right identifications, as for example using more than two MS/MS transitions, analysing samples by HR MS techniques, and/or improving the chromatographic separation.

The goal of this paper is, firstly, to develop a rapid, accurate and sensitive analytical methodology based on the use of LC-MS/MS QqQ for the simultaneous determination of 21 analytes, including seven parent compounds and their main metabolites. Then, validation has

been performed in a high number of water samples (six different surface water and six different effluent wastewater) trying to cover quite distinct sample compositions and situations that can appear when analyzing real samples. Finally, the method has been applied to environmental water samples to test its applicability to investigate the presence of these compounds in real-world scenarios.

2. Experimental

2.1. Reagents and chemicals

Reference standards of pharmaceuticals were purchased from Sigma-Aldrich (St Louis, MO, USA). Reference standards of metabolites were obtained from Toronto Research Chemicals (Ontario, Canada), with the exception of carbamazepine 10,11-epoxide, enalaprilat, 4-aminoantipyrine and clofibric acid, which were also supplied by Sigma-Aldrich. Their chemical structures are shown in Figure S1 (Supporting Information).

Isotopically labelled compounds used as ILIS (omeprazole-d_3, enalaprilat-d_5 and carbamazepine 10,11-epoxide-d_{10}) were from CDN Isotopes (Quebec, Canada).

Individual stock solutions of pharmaceuticals/metabolites (around 500 mg/L) were prepared dissolving an accurately weighted amount in methanol. The individual stock solutions were mixed and diluted with methanol to give a final concentration of around 1 mg/L (40% MeOH, 60% HPLC-grade water, approximately). This solution was subsequently diluted with HPLC-grade water to obtain working mixed solutions of pharmaceuticals/metabolites. These solutions were used for spiking samples in the validation study and also for preparation of calibration standards, which were prepared in methanol-water (10:90, v/v).

Individual stock solutions of ILIS were also prepared in methanol. Mix working solutions at 5 µg/L (for surface water (SW) samples) or at 50 µg/L (for effluent wastewater (EWW) samples) were prepared in HPLC-grade water and used as surrogates.

2.2. Liquid chromatography-tandem mass spectrometry

Ultra high pressure liquid chromatography (UHPLC) analysis was carried out using an Acquity UPLC (Waters Corp., Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatographic separation was performed using an Acquity UPLC HSS T3 C18 column, 1.8 µm, 100 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL/min. The column was kept at 50 ºC and the samples at 5 ºC. Mobile phase consisted of a water/methanol 0.01% HCOOH gradient, where the organic percentage was changed linearly as follows: 0 min, 10%; 12 min, 80%; 12 min, 80%; 12.1 min; 10%. Analysis run time was 13 min. The sample injection volume was 100 µL (full loop). A TQD (triple quadrupole) mass spectrometer with an orthogonal Z-spray-electrospray interface (Waters Corp., Milford, MA, USA) was used. For more details, see Supporting Information.

2.3. Validation study

Method accuracy (expressed as percentage recovery) and precision (expressed as repeatability in terms of relative standard deviation, RSD) were estimated by means of recovery experiments in 12 different samples spiked at various concentrations (0.02 µg/L in SW; 0.1 and 0.4 µg/L in EWW). SW samples used for validation were collected in different sites of the Mediterranean Spanish area of Valencia (Mijares and Jucar rivers, Sitjar and Mª Cristina reservoirs, Clot de Burriana lake and coastal lagoon Albufera de Valencia). EWW samples were collected from different WWTPs of the same area. For each individual sample, recovery experiments were performed by triplicate, giving a total number of 18 data for SW and 18 for EWW at each spiked concentration.

For each sample under study, the limit of quantification (LOQ) was estimated for a signal-to-noise ratio (S/N) of 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. In this way, average LOQ and the interval (both in ng/L) were estimated for both SW and EWW samples**.** True blank samples were not found for several analytes, as they were already present in the samples tested. In these cases, LOQs were estimated from the analyte levels quantified in the non-spiked “blanks”. The instrumental limit of detection (LOD) was estimated for S/N = 3 from the chromatograms of the standard at the lowest concentration level tested in the calibration curve.

Linearity of the method was studied by analyzing standard solutions in triplicate at seven concentrations from 0.25 to 25 μg/L (equivalent to 0.005-0.5 μg/L in the water sample). Satisfactory linearity using least squares regression was assumed when the correlation coefficient (r) was higher than 0.99 and residuals lower than 30% without significant trend, based on absolute responses, except for those compounds that were quantified with ILIS (relative responses).

2.4. Recommended procedure

50 mL water sample was spiked with the corresponding ILIS mix working solution, giving a final concentration of 0.5 μg/L (surface water) and 5 μg/L (effluent wastewater) for each individual ILIS. Oasis HLB (60 mg) cartridges used for SPE were previously conditioned with 6 mL MeOH and 6 mL HPLC-grade water. Then, the samples were passed through the cartridge by gravity and, after drying under vacuum for 15 minutes, analytes were eluted with 5 mL MeOH. The extract was evaporated to dryness under a gentle nitrogen stream at 40ºC and reconstituted with 1 mL MeOH–water (10:90, v/v). Finally, 100 μL were injected into the UHPLC–MS/MS system under the conditions shown in Table 1.

Quantification was made by calibration standards in solvent, using relative responses analyte/ILIS, or absolute responses, depending whether ILIS was used for correction or not.

Table 1
MS/MS optimized conditions for selected compounds.

Compound	ESI	Cone (V)	Q Transition (Q)	C.E. (eV)	q1 Transition	C.E. (eV)	q2 Transition	C.E. (eV)	Q/q1[a]	Q/q2[a]	LOD (pg)
Carbamazepine	+	25	237.3 > 194.2	25	237.3 > 179.2	35	237.3 > 165.2	40	4.8	7.1	0.3
Carbamazepine 10,11-epoxide	+	15	253.4 > 180.2	20	253.4 > 236.2	10	253.4 > 210.3	20	1.7	10.7	0.9
10,11- Dihydro-10,11-dihydroxy carbamazepime	+	15	271.0 > 180.1	30	271.0 > 253.0	5	271.0 > 236.0	10	1.5	1.7	1.6
Clarithromycin	+	40	748.3 > 158.1	30	748.3 > 83.0	50	590.3 > 158.1[b]	25	2.2	2.4	0.3
N-Desmethyl clarithromycin	+	25	735.0 > 144.2	20	735.0 > 576.8	20	737.0 > 146.2	15	7.6	168.1	0.4
Clopidogrel	+	25	322.0 > 212.0	15	322.0 > 184.0	25	324.0 > 214.0	15	1.5	3.1	0.3
Clopidogrel carboxylic acid	+	20	308.3 > 198.2	15	308.3 > 77.0	45	310.3 > 200.2	10	1.0	4.1	0.9
Enalapril	+	35	377.4 > 91.1	55	377.4 > 234.2	20	377.4 > 160.2	30	2.1	4.6	1.1
Enalaprilat	+	30	349.5 > 91.1	50	349.5 > 206.3	20	349.5 > 117.2	35	2.0	2.5	10.2
Losartan	+	20	423.2 > 207.0	25	423.2 > 405.2	15	425.2 > 207.0	25	3.0	9.0	0.4
Losartan carboxylic acid	+	25	437.2 > 207.1	30	437.2 > 235.0	20	439.2 > 207.2	20	0.5	1.6	5.7
Omeprazole	+	30	346.3 > 198.1	10	346.3 > 136.1	35	346.3 > 151.1	20	0.9	2.0	2.6
4-Hydroxy omeprazole sulfide	+	20	316.4 > 168.2	25	316.4 > 149.2	25	316.4 > 136.2	25	1.0	1.0	0.5
5-Hydroxy omeprazole	+	15	362.1 > 152.1	35	362.1 > 214.0	20	362.1 > 196.2	30	1.7	3.2	0.5
Sulfamethoxazole	+	40	254.0 > 64.9	50	254.0 > 91.9	30	254.0 > 155.9	20	1.2	49.4	0.7
N-Acetyl sulfamethoxazole	+	30	296.0 > 134.2	20	296.0 > 198.0	20	-	-	2.8	-	0.7
4-Acetamido antipyrine	+	20	246.4 > 83.1	25	246.4 > 228.3	15	246.4 > 104.1	20	1.6	1.6	1.2
4-Amino antipyrine	+	20	204.4 > 56.1	15	204.4 > 83.1	15	204.4 > 94.1	15	5.9	5.6	1.0
4-Formylamino antipyrine	+	25	232.4 > 56.1	25	232.4 > 83.1	20	232.4 > 104.1	20	1.6	1.0	2.9
Clofibric acid	-	20	213.3 > 127.0	15	215.2 > 129.0	10	213.3 > 85.1	10	1.1	5.3	23.2
Fenofibric acid	+	25	319.0 > 233.0	15	319.0 > 138.9	30	319.0 > 121.0	30	1.2	2.6	1.2
Carbamazepine 10,11-epoxide-d_{10}	+	20	263.1 > 190.0	20	-	-	-	-	-	-	-
Enalaprilat-d_5	+	25	354.1 > 211.1	20	-	-	-	-	-	-	-
Omeprazole-d3	+	30	349.3 > 198.1	10	-	-	-	-	-	-	-

ESI, electrospray ionization; Q, quantification; q, confirmation; C.E., collision energy.
[a]Average for seven standards, from 0.25 to 25 mg/L.
[b]In this case an in-source fragment was used as precursor ion and the cone voltage was 55 V.
In bold the parent pharmaceuticals, in italics the metabolites and with regular format, the ILIS used.

3. Results and discussion

In a previous work[13], five pharmaceutical metabolites were identified in urban wastewater samples by UHPLC-QTOF MS, after detection of the parent pharmaceuticals and subsequent data re-evaluation in a retrospective way. These compounds were N-desmethyl clarithromycin, fenofibric acid, clopidogrel carboxylic acid and 4-hydroxy omeprazole sulfide. Analysis by QTOF also allowed us to discover the presence of several metabolites of the analgesic dipyrone in urban wastewater[17]. Based on these previous findings, we decided to widen the study of pharmaceutical metabolites and to develop a multi-residue sensitive method based on LC-MS/MS with triple quadrupole for the simultaneous quantification and confirmation of some relevant metabolites. In addition to those compounds previously detected in our previous works, the list of target metabolites was completed with nine compounds that were reported to be present in SW and EWW[9,11,18] and taking into account their commercial availability as reference standards. Moreover, the parent pharmaceuticals of the metabolites selected were also included in the method as they might not be completely metabolized[1,3]. The parent compounds clofibrate, fenofibrate and dipyrone were not considered because they are pro-drugs[1,20] and therefore, they are not expected to be found in the water samples.

3.1. MS and MS/MS optimization

Three SRM transitions were selected for each compound to assure the reliable identification of the compounds detected in water samples. The most sensitive transition was used for quantification (Q) whereas the other two were used for confirmation (q_1 and q_2). For N-acetyl sulfamethoxazole only one confirmation transition could be monitored due to its poor fragmentation. Mass spectrometry parameters, precursor and product ions selected, instrumental LODs and ion ratios (Q/q) used for confirmation are shown in Table 1. For further details, see Supporting Information.

3.2. Chromatographic optimization

In order to optimize chromatographic separation, both methanol and acetonitrile solvents with different HCOOH and NH_4Ac contents were evaluated. Acetonitrile was discarded because sensitivity decreased for most compounds in comparison with MeOH. Regarding the modifiers, the use of NH_4Ac led to worse sensitivity compared with HCOOH. Besides, the

addition of HCOOH favoured the retention of acidic compounds in the LC column, such as losartan carboxylic acid or clofibric acid. Thus, for losartan, the retention time shifted from 6.28 min (NH_4Ac 5 mM) to 9.19 min (HCOOH, 0.01%). This behaviour was similar for clofibric acid (from 6.62 min to 9.06 min). On the contrary, for a few analytes, the chromatographic run time decreased when HCOOH was added, especially for clarithromycin, which eluted the latest. However, enalapril and its metabolite presented a worse peak shape when acid was present in the mobile phase. This controversial situation is quite usual when compounds with very different physico-chemical characteristics are simultaneously analysed, and obviously a compromise has to be reached.

Once the mobile phase was selected (water/methanol 0.01% HCOOH), two UHPLC C18 columns were compared (HSS T3 and BEH, both 10 cm). The results were similar showing that both columns are suitable for the retention of a broad group of compounds with different polarity. Finally, HSS T3 column was selected because the analytes were more retained and peak shape was better (narrower peaks) for a few compounds such as losartan carboxylic acid and enalaprilat.

After testing several volumes (20, 50 and 100 µL), the optimal injection volume was optimized, selecting 100 µL due to the increased sensitivity without affecting the peak shape. Column temperature was maintained at 50 ºC to improve peak shape of enalapril[21].

3.3. Solid phase extraction optimization

Metabolites are usually more polar than parent compounds, making their simultaneous extraction more problematic. Therefore, the optimization of the SPE step is especially important in this case. In this work, the extraction efficiency of three cartridges was checked (Oasis HLB (200 mg), Oasis MCX (150 mg) and Oasis MAX (150 mg), using HPLC-grade water spiked with the analytes. Oasis HLB can be used for a wide range of target compounds with quite distinct polarities, while MCX is suitable for compounds with basic groups and MAX for acidic compounds. Oasis HLB was tested at neutral pH, while MCX required acidification of the water sample (pH 2) to ensure protonation of basic compounds, and MAX required working at basic medium (pH 11) in order to fully deprotonate acidic compounds. After loading the samples, the cartridges were dried under vacuum for 15 min. The elution was carried out with 8 mL MeOH

(HLB), with 4 mL MeOH followed by 4 mL MeOH 5% NH_4OH (MCX), or with 4 mL MeOH followed by 4 mL MeOH 5% HCOOH (MAX).

Our results showed that recoveries for N-desmethyl clarithromycin and carbamazepine 10,11-epoxide were lower using MCX (around 30%). The first one is expected to be efficiently retained on MCX cartridge due to the protonation of the amino group. A possible explanation for these unexpected results might be that the elution with 4 mL 5% NH_4OH was not enough to break its strong retention in the cartridge, yielding to poor recoveries. On the contrary, the acidification of the water sample would generate the diol group through the epoxide ring opening of carbamazepine 10,11-epoxide, being partially converted into 10,11-dihydro-10,11-dihydroxy carbamazepine, and leading to low SPE recoveries.

In general, recoveries with HLB and MAX were quite similar, although the first one showed more reproducible figures for all compounds (data not shown). Therefore, HLB cartridges were selected for subsequent experiments. Then, a comparison between Oasis HLB containing 60 mg and 200 mg was carried out, eluting with 5 and 8 mL MeOH, respectively. As similar results were obtained for all compounds, Oasis HLB 60 mg was selected to economize the amount of stationary phase. Finally, this cartridge was tested adjusting the pH of the sample loaded at three values (pH 3, 7 and 9). As a compromise, neutral pH was selected for sample extraction, as nearly all compounds showed satisfactory recoveries (between 70 and 120%), with the exception of 4-aminoantipyrine (4-AA) and enalaprilat (recoveries around 40%).

3.4. Method validation

The linearity of the method was satisfactory between 0.25 - 25 μg/L for all compounds. These values corresponded to 0.005-0.5 μg/L in the water sample, taking into account the 50-fold pre-concentration factor applied along the sample procedure. For validation purposes, each of the 12 water samples selected (6 SW and 6 EWW) were spiked at different concentration levels (0.02 μg/L in SW; 0.1 and 0.4 μg/L in EWW). Experiments were performed by triplicate for each spiked sample. Recoveries were determined by comparing the concentration obtained after applying the recommended procedure with the nominal concentration of the spiked samples, performing quantification by standards calibration in solvent. “Blank” samples, containing only the ILIS mix, were also processed to subtract the concentration of the target analyte when it was present in the sample used in the recovery experiments.

The method was tested at 0.02 μg/L in the surface water samples (recoveries shown in Table S1, Supporting Information). A few compounds could not be properly validated in one of the samples tested (Jucar river) due to the high analyte concentration found in the "blank" sample. Enalaprilat and clofibric acid could not be validated in some samples due to the low sensitivity observed for these compounds, which would have required higher spiking levels to be validated. With very rare exceptions, data were satisfactory (between 70% and 120%) for most of the compounds. In a few cases, recoveries varied significantly from one sample to another. This was the case of clarithromycin (individual recoveries between 52 - 107%). This variation might be explained by the distinct matrix effects that particularly affected to this compound and that varied notably from one water sample to another.

Clopidogrel and 4-aminoantipyrine presented low recoveries (around 45%) in all samples. In order to know whether poor recoveries were due to matrix effects or to poor extraction in the SPE cartridge, for each individual SW sample the extract obtained after SPE was spiked and the analyte responses compared with standards in solvent at the same concentration (data not shown). Our results showed that low clopidogrel recoveries were due to matrix effects (signal suppression). On the contrary, no relevant matrix effects were observed for 4-aminoantipyrine; consequently, its low recoveries were attributed to losses during the SPE process. On the other hand, four compounds showed recoveries above 120% (losartan carboxylic acid; 10,11-dihydro-10,11-dihydroxy carbamazepine; omeprazole; 5-hydroxy omeprazole) due to matrix signal enhancement. In the case of omeprazole, matrix effects could be corrected by using its own ILIS, obtaining satisfactory recoveries in all SW samples.

In total, three ILIS were used in this work and tested for matrix effects correction. In addition to the above mentioned omeprazole, two more compounds were corrected with their own ILIS (carbamazepine 10,11-epoxide and enalaprilat). It is interesting to notice that the ILIS carbamazepine 10,11-epoxide-d_{10} did not fully correct matrix effects for its own analyte. Similar situation has been reported for some labelled compounds with a high degree of deuterated atoms[22]. It seems that the isotope-labelled compound and the analyte had different physico-chemical behaviour, leading to an (unexpected) unsatisfactory correction. A possible explanation could be related to different hydrolysis kinetics between labelled and unlabelled epoxide metabolites caused by the presence of deuteriums in the hydrolysis site.

Regarding EWW, notably matrix effects are normally expected. In previous works[23,24] the use of ILIS was the alternative chosen to compensate for matrix effects in pharmaceutical

analysis in water. When the analyte ILIS is not available, the use of an analogue might be satisfactory, although commonly it can not ensure appropriate correction for all analyte/water sample combinations[24]. In the present work, the availability of only three ILIS made the correction of matrix effects problematic. Sample dilution might be a good alternative, also simple and fast, if sufficient sensitivity is achieved by the analytical method. After testing different dilutions of sample with HPLC-grade water, a 4-fold dilution was found to be adequate for accurate quantification, also maintaining a satisfactory sensitivity. Spiking levels tested in the non-diluted effluent wastewaters from different WWTPs were 0.1 and 0.4 μg/L (i.e. 0.025 and 0.1 μg/L in the 4-diluted samples). In general, recoveries and precision were satisfactory for most compounds at both fortification levels (Table S2 in Supporting Information). Two metabolites of dipyrone (4-acetamidoantipyrine and 4-formylantipyrine) could not be validated in some samples due to the high concentrations found in the "blanks". Low recoveries were obtained for clofibric acid, fenofibric acid and clopidogrel at both levels due to signal suppression that could not be compensated by sample dilution (x 4). On the contrary, some analytes (e.g. losartan carboxylic acid, N-acetyl sulfamethoxazole, 5-hydroxy omeprazole or enalapril) yielded values above 120% in several samples. This behaviour was also observed in SW, as previously commented.

Enalaprilat could not be validated at the lowest level assayed (0.1 μg/L) due to its low sensitivity. At the highest concentration (0.4 μg/L), the average recovery was 66%, which could be improved to 108% by correction of matrix effects thanks to the availability of ILIS enalaprilat-d_5. As occurred in SW, the use of ILIS carbamazepine 10,11-epoxide-d_{10} did not fully correct matrix effects for its own analyte leading to recoveries of 45% and 74% at the low and high spiking concentrations, respectively.

It is worth to notice the case of omeprazole, which could not be validated in EWW probably due to its low stability. In order to evaluate the stability of the omeprazole standard, an individual new solution of this pharmaceutical was prepared, and injected in the LC-MS/MS instrument every week. As can be seen in Figure 1a, the omeprazole signal notably decreased along the time, while in parallel the concentration of 4-hydroxy omeprazole sulfide, which was not included in the standard solution, increased (Figure 1b). Thus, it seems clear that omeprazole was unstable in aqueous solution and was transformed to 4-hydroxy omeprazole sulfide. The degradation of omeprazole to the sulfide derivative in HPLC-grade water and kept in dark has been previously reported in literature[25]. In our study, the transformation started to be

more evident after 14 days of storage in the cooler (-4 ºC, darkness). Thus, to avoid a wrong quantification for omeprazole and 4-hydroxy omeprazole sulfide when analyzing real samples, standards should be renewed weekly.

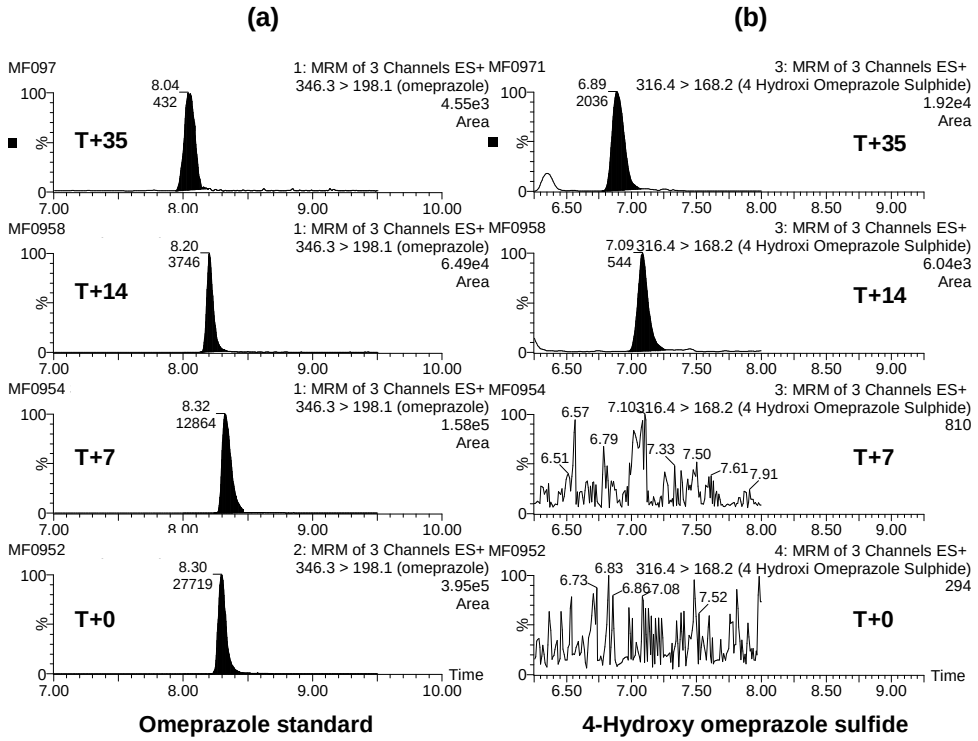

Figure 1. LC-MS/MS chromatograms for (a) omeprazole reference standard (100 μg/L) injected different days after preparation of the standard solution (b) 4-hydroxy omeprazole sulfide formed by degradation of omeprazole**.**

LOQs were estimated for every water sample tested (i.e., 6 SW and 6 EWW). For SW, average LOQs ranged from 0.4 to 7.7 ng/L (Table S1, Supporting Information). The two exceptions were clofibric acid and enalapril, which presented low sensitivity, with the result of higher LOQs (around 25 ng/L in SW). With the exception of enalaprilat, average LOQs for EWW varied from 1.8 to 145 ng/L: for 3 compounds, LOQs were < 6 ng/L, and for another 10 analytes they were lower than 60 ng/L (Table S2). As can be seen, in several cases the LOQs varied notably from one sample to other. This highlights once more that matrix effects can be rather different from one sample to another. Therefore, giving an LOQ value estimated just from a

given sample, as reported in most of papers, might not be realistic. Consequently, LOQ reported should be taken with precaution, as the situation may be rather different when applying the method to a set of real-world samples. Concerning instrumental LODs, they ranged from 0.3 to 10 pg, except for clofibric acid, the only compound measured in negative ionization mode (Table 1).

3.5. Application to water samples

The developed methodology was applied to the analysis of 12 SW samples collected at selected sites from the Spanish Mediterranean area of Valencia. 12 wastewater samples, consisting on 24-h composite urban effluent wastewater samples, were collected from different WWTPs located in the same area.

In every sequence of sample analysis, the calibration curve was injected twice, at the beginning and the end of the sample batch. Moreover, quality control samples (QCs) were included in the sample sequence. QCs consisted on SW or EWW samples spiked at the LOQ level. They were prepared randomly selecting one of the water samples analyzed within the batch, following the same analytical procedure than for the samples. In the case that the sample used for QC preparation contained any of the compounds analyzed, the concentration calculated in the sample was subtracted from that calculated in the spiked sample.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q_1 and q_2) transitions. As three transitions were acquired, two intensity ion-ratios could be used for confirmation of the identity. The finding was considered as positive when the experimental ion-ratios were within the tolerance range[26] and the retention time in the sample within ± 2.5% the retention time of the reference standard.

All parent compounds, except omeprazole, were detected in the surface water samples at least once (Table 2). Regarding metabolites, three of them (4-aminoantipyrine, clofibric acid and enalaprilat) were never found, while the rest were commonly detected in the samples. The highest concentrations corresponded to the dipyrone metabolites 4-acetamidoantipyrine and 4-formylaminoantipyrine (0.89 and 0.87 µg/L, respectively). Dipyrone is a pro-drug widely used as antipyretic. Its main metabolites have been previously investigated, and high concentrations reported in wastewaters[14,27].

Table 2
Summary of the results obtained in the analysis of water samples.

Compounds	SW (n = 12)				EWW (n = 12)			
	Min Level (μg/L)	Max Level (μg/L)	Median (μg/L)	% positive findings	Min Level (μg/L)	Max Level (μg/L)	Median (μg/L)	% positive findings
Carbamazepine	0.002	0.026	0.009	58	0.43	2.76	1.01	100
Carbamazepine 10,11-epoxide	< LOQ	< LOQ	< LOQ	17	n.d.	n.d.	n.d.	-
10,11- Dihydro-10,11-dihydroxy carbamazepine	0.004	0.374	0.048	92	0.55	6.85	1.76	100
Clarithromycin	< LOQ	0.012	0.004	67	0.04	0.39	0.09	67
N-Desmethyl clarithromycin	< LOQ	0.004	0.004	50	0.04	0.15	0.09	100
Clopidogrel	< LOQ	0.002	0.002	100	0.006	0.022	0.008	100
Clopidogrel carboxylic acid	0.002	0.012	0.003	67	0.09	0.61	0.20	100
Enalapril	n.d.	< LOQ	< LOQ	8	n.d.	n.d.	n.d.	-
Enalaprilat	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	-
Losartan	0.003	0.099	0.004	67	0.03	0.37	0.29	100
Losartan carboxylic acid	<LOQ	0.116	0.009	67	0.01	1.56	0.49	100
Omeprazole	n.d.	n.d.	n.d.	-	n.d.	n.d.	n.d.	-
4-Hydroxy omeprazole sulfide	<LOQ	0.028	< LOQ	33	0.06	0.29	0.11	100
5-Hydroxy omeprazole	0.004	0.004	0.004	8	0.12	0.25	0.22	58
Sulfamethoxazole	< LOQ	< LOQ	< LOQ	8	0.05	1.86	0.11	100
N-Acetyl sulfamethoxazole	< LOQ	< LOQ	< LOQ	33	0.03	0.96	0.09	100
4-Acetamido antipyrine	0.002	0.894	0.068	75	0,49	7.91	3.20	100
4-Amino antipyrine	n.d.	n.d.	n.d.	-	0.53	7.98	0.85	25
4-Formylamino antipyrine	0.037	0.871	0.079	67	0.98	5.76	3.23	100
Clofibric acid	n.d.	nd	n.d.	-	n.d.	n.d.	n.d.	-
Fenofibric acid	0.008	0.182	0.020	33	0.01	0.36	0.23	100

Median concentrations for most of the compounds in surface waters were lower than 0.01 µg/L. The metabolite 10,11-dihydro-10,11-dihydroxy carbamazepine was however present at higher concentrations. This compound was found in 92% of the samples in contrast to the other carbamazepine metabolite studied in this work (carbamazepine 10,11-epoxide), which was only present in 17% of the samples. This is in accordance with the metabolism of carbamazepine. This pharmaceutical, used for the treatment of epilepsy, schizophrenia and bipolar disorder, undergoes extensive metabolism by cytochrome P450 system in the liver. Carbamazepine seems not to be efficiently removed in WWTPs, which may explain its frequent detection in environmental samples[6,28]. Also in agreement with our findings is the fact that the most relevant metabolite 10,11-dihydro-10,11-dihydroxy carbamazepine was widely detected in waters, and to a lesser extent the metabolite carbamazepine 10,11-epoxide[18]. It is worth to notice that, most of papers dealing with the presence of carbamazepine and metabolites in the aquatic environment only take into account carbamazepine 10,11-epoxide[6,7,29,30], despite that higher concentrations are commonly found for 10,11-dihydro-10,11-dihydroxy carbamazepine[18]. This might be due to the fact that the epoxide is the active metabolite of carbamazepine[18].

In relation to effluent wastewater samples, 13 out of 21 compounds were detected in 100% of the samples, which illustrates the ubiquity of these compounds in the wastewaters. All parent compounds, except omeprazole and enalapril, were frequently detected. In the case of omeprazole, despite being one of the most consumed pharmaceuticals in Spain, it was not detected in any of the samples. On the contrary, the two omeprazole metabolites included in this study were detected, and one of them (4-hydroxy omeprazole sulfide) was found in all the samples analyzed (Table 2). This is in agreement with our previous research on omeprazole metabolism and on its transformation products in water[31,32]. Enalapril and enalaprilat (its active metabolite) were not detected in any of the samples. Both compounds are frequently found in influent wastewater[11,21] but they are much less ubiquitous in EWW.

Sulfamethoxazole and its N-acetyl derivate were found in all EWW samples at similar average concentration level. Despite the derivative does not have pharmacological activity, it however presents ecotoxicity[7].

The highest concentrations were by far for dipyrone metabolites, with maximum concentrations around 8 µg/L, for both 4-acetamidoantipyrine and 4-aminoantipyrine, while 4-formylaminoantipyrine reached a maximum value around 6 µg/L. The later metabolite was

present in 25% of the samples while the other two were found in all the effluent wastewaters analyzed.

Similarly, the concentrations of the pharmaceuticals losartan, carbamazepine and especially for clopidogrel were lower than for their metabolites. As occurred in surface waters, high levels of 10,11-dihydro-10,11-dihydroxy carbamazepine were found due to the extensive metabolism of carbamazepine.

Regarding the pro-drugs compounds, only fenofibric acid was detected while clofibric acid was not found in any sample. It seems that this compound suffers some kind of transformation/degradation in the sewer system and/or in the aquatic environment, which makes its detection in water samples unlikely.

Illustrative UHPLC-MS/MS chromatograms are shown in Figure 2 for a EWW sample that was positive for up to 16 compounds, with concentrations varying from 0.02 μg/L (clopidogrel) to 7.98 μg/L (4-aminoantipyrine).

In summary, the application of this method to surface and effluent urban wastewater samples showed that 12 out of 14 metabolites were present in a notable number of samples analyzed. Interestingly, metabolite concentrations were on the same order or even higher than those of the parent compounds. This illustrates the importance of including metabolites and transformation products in the methods applied for water analysis in order to have a wider and more realistic knowledge on the occurrence and fate of pharmaceuticals in the aquatic environment.

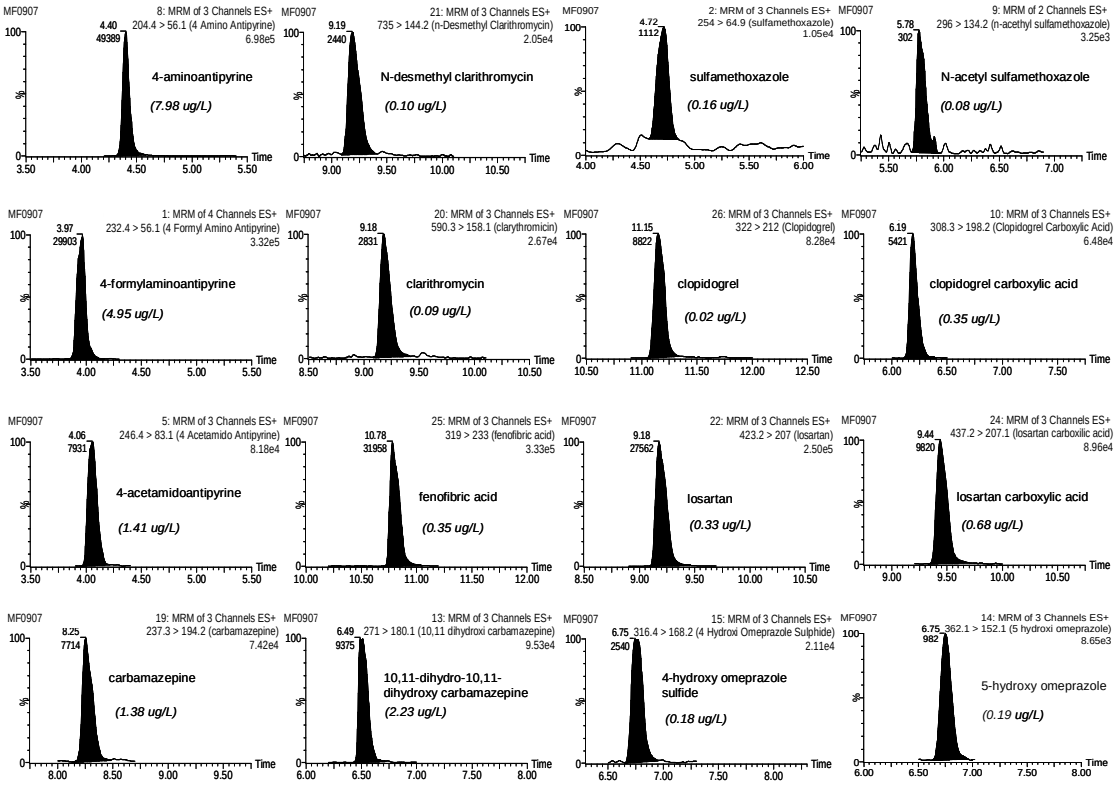

Figure 2. LC–MS/MS chromatograms (Q transition) for an effluent wastewater that was positive to 16 compounds (5 pharmaceuticals and 11 metabolites).

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Supporting Information

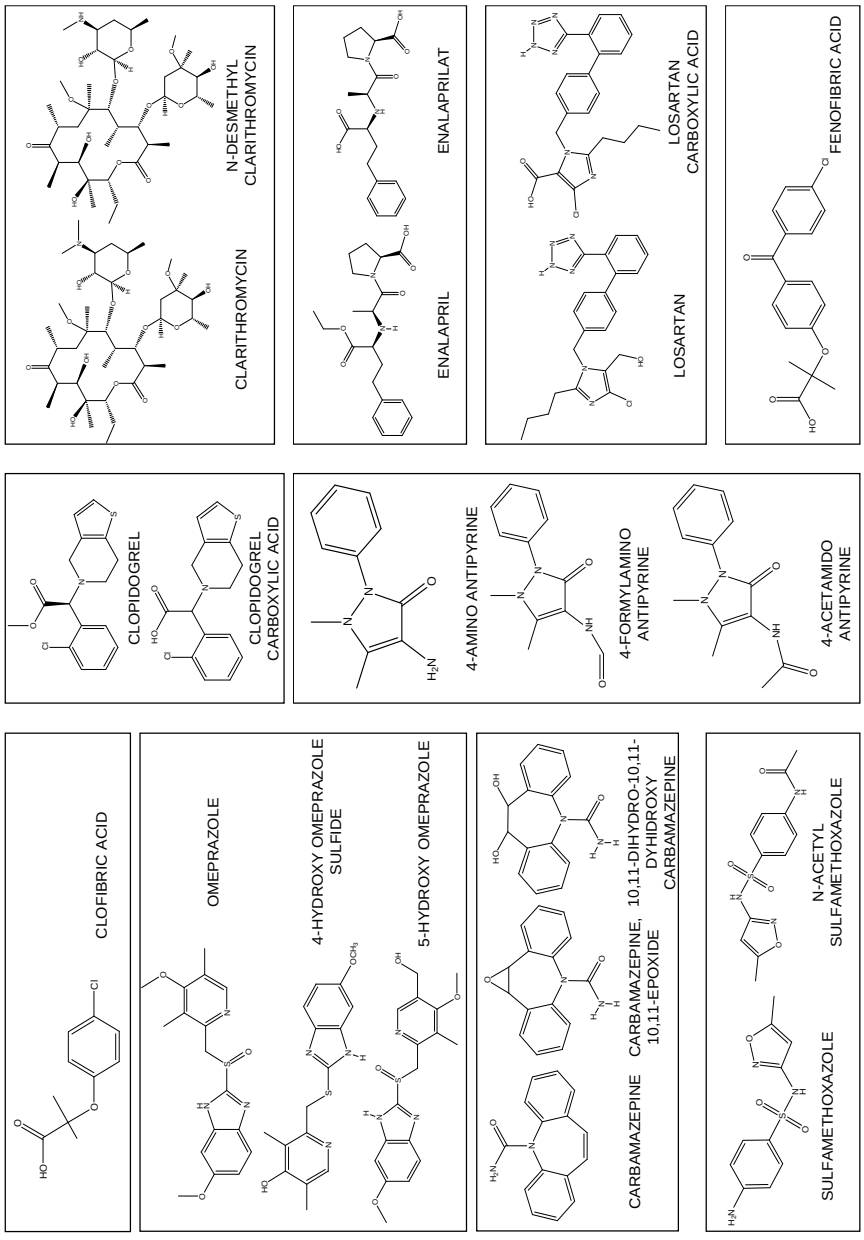

Figure S1. Structures of the selected compounds.

Table S1

Method validation for surface water. Recovery (%) and relative standard deviation (RSD, %) for six different SW samples spiked at 0.02 µg/L. Each sample was analyzed in triplicate.

Compound	t_R (min)	Recovery (%)						Average recovery (%)	Average LOQ (ng/L)	LOQ range (ng/L)
		Sitjar reservoir	M[a] Cristina reservoir	Clot lake	Jucar river	Mijares river	Albufera lake			
Carbamazepine	8.32	72	59	85	90	99	56	77 (22)	0.6	0.3 – 0.7
Carbamazepine 10,11-epoxide	6.94	106	99	67	94	110	61	89 (23)	2.7	1.7 – 3.7
Carbamazepine 10,11-epoxide*		39	55	70	90	57	63	62 (27)		
10,11- Dihydro-10,11-dihydroxy carbamazepine	6.57	177	217	177	a	140	127	168 (21)	2.2	1.4 – 3.8
Clarithromycin	9.20	107	100	52	53	64	66	74 (32)	1.9	1.3 – 2.6
N-Desmethyl clarithromycin	9.24	101	88	69	69	73	53	75 (22)	2.5	0.6 – 4.4
Clopidogrel	11.23	50	40	52	39	38	36	43 (16)	0.4	0.2 – 0.7
Clopidogrel carboxylic acid	6.25	82	101	101	50	121	94	91 (26)	1.1	0.5 – 2.0
Enalapril	7.45	80	111	91	103	105	155	107 (24)	22.8	8.9 – 47.6
Enalaprilat	4.93	b	b	b	b	b	b	b	-	-
Enalaprilat*		b	b	b	b	b	b	b		
Losartan	9.23	105	109	104	101	128	131	113 (12)	1.3	0.6- 2.8
Losartan carboxylic acid	9.50	120	130	121	108	162	153	132 (16)	5.4	1.7 – 7.5
Omeprazole	7.99	161	237	167	207	176	345	215 (32)	2.4	2.2 – 2.8
Omeprazole*		102	68	70	87	103	93	87 (18)		
4-Hydroxy omeprazole sulfide	6.83	88	102	84	84	80	75	85 (11)	2.3	1.9 – 2.7
5-Hydroxy omeprazole	6.82	123	145	149	150	131	156	142 (9)	3.0	2.2 – 3.3
Sulfamethoxazole	4.79	98	77	112	106	112	70	96 (19)	7.7	4.0 – 24.8
N-Acetyl sulfamethoxazole	5.90	120	95	128	97	143	95	113 (18)	3.9	2.0 – 7.1
4-Acetamido antipyrine	4.09	87	116	114	a	122	66	101 (23)	3.0	0.9 – 6.6
4-Amino antipyrine	4.42	44	51	76	a	33	52	51 (31)	1.6	0.6 – 3.5
4-Formylamino antipyrine	3.99	95	136	111	a	142	103	117 (18)	2.7	1.3 – 5.5
Clofibric acid	9.45	108	90	83	62	b	b	86 (22)	24.5	17.1 – 30.6
Fenofibric acid	10.86	80	61	83	a	67	101	78 (20)	1.9	1.2 – 2.5

a: Not estimated due to the high analyte levels found in the “blank” sample; b: Not estimated due to the poor sensitivity.

* Recoveries calculated using their own ILIS

In bold the parent pharmaceuticals and with regular format, the metabolites.

Table S2

Method validation for effluent wastewater. Recovery (%) and relative standard deviation (RSD%) for six different EWW samples spiked at 0.1 and 0.4 µg/L
Each sample was analyzed in triplicate.

Compounds	Recovery (%) at 0.1 µg/L						Average	Recovery (%) at 0.4 µg/L						Average	Average LOQ (ng/L)	LOQ range (ng/L)
	EWW 1	EWW 2	EWW 3	EWW 4	EWW 5	EWW 6		EWW 1	EWW 2	EWW 3	EWW 4	EWW 5	EWW 6			
Carbamazepine	118	90	112	114	89	113	106 (12)	125	115	87	111	100	110	108 (12)	17.3	7.5 – 31.3
Carbamazepine 10,11-epoxide	107	119	116	107	107	118	112 (5)	137	137	96	144	137	131	130 (13)	36.1	21.6 – 56.3
Carbamazepine 10,11-epoxide*	35	57	41	42	41	53	45 (19)	80	78	58	79	79	71	74 (12)		
10,11- Dihydro-10,11-dihydroxy carbamazepine	96	97	111	a	70	88	92 (16)	126	118	91	134	137	142	125 (19)	144.9	71.1 – 287.1
Clarithromycin	121	139	115	119	135	127	126 (8)	104	95	62	98	111	67	90 (22)	13.5	3.5 – 24.7
N-Desmethyl clarithromycin	97	107	89	102	106	92	99 (8)	74	71	57	72	90	57	70 (17)	13.8	4.6 – 19.4
Clopidogrel	29	31	32	27	39	41	33 (17)	53	55	53	61	46	53	53 (9)	1.8	0.7 – 2.7
Clopidogrel carboxylic acid	99	114	136	96	102	119	111 (14)	113	124	111	108	99	91	108 (11)	15.7	10.1 – 21.8
Enalapril	138	153	136	149	141	156	146 (6)	158	149	146	163	107	171	149 (15)	132.7	85.0 -179.7
Enalaprilat	b	b	b	b	b	b	b	89	68	70	70	59	42	66 (23)	513	348 - 693
Enalaprilat*	b	b	b	b	b	b	b	105	117	80	106	106	132	108 (16)		
Losartan	96	109	92	96	111	124	105 (12)	116	111	68	109	103	103	102 (17)	5.9	3.2 – 13.0
Losartan carboxylic acid	131	153	182	183	149	207	168 (17)	197	166	113	165	159	132	155 (19)	44.5	25.2 – 77.5
Omeprazole	c	c	c	c	c	c	c	c	c	c	c	c	c	c	-	-
Omeprazole*	c	c	c	c	c	c	c	c	c	c	c	c	c	c		
4-Hydroxy omeprazole sulfide	94	123	91	107	114	81	102 (16)	104	120	94	105	121	97	107 (11)	37.2	19.6 – 60.1
5-Hydroxy omeprazole	103	132	145	132	108	117	123 (13)	137	139	124	137	129	123	131 (5)	33.9	9.5 – 60.1
Sulfamethoxazole	71	59	134	100	108	115	98 (29)	92	110	74	97	103	100	96 (13)	64.3	35.1 – 77.9
N-Acetyl sulfamethoxazole	73	143	112	112	154	140	123 (24)	112	129	119	115	128	118	120 (6)	56.9	32.4 – 80.8
4-Acetamido antipyrine	a	a	a	a	a	83	83	a	a	a	a	a	106	106	60.3	60.3
4-Amino antipyrine	55	64	64	49	a	a	58 (13)	47	48	88	59	a	59	60 (28)	130.8	54.8 – 310.1
4-Formylamino antipyrine	149	a	a	118	a	a	134 (16)	216	222	a	109	179	a	182 (29)	129.5	84.9 – 189.3
Clofibric acid	51	51	30	51	46	40	45 (19)	51	52	41	29	29	26	38 (30)	106.8	78.7 – 160.0
Fenofibric acid	47	65	41	58	65	67	57 (19)	65	65	52	44	59	59	58 (14)	5.9	4.7 – 8.5

a: Not estimated due to the high analyte levels found in the “blank” sample.; b: Not estimated due to the poor sensitivity; c: Not estimated due to the standard degradation.

* Recoveries calculated using their own ILIS

In bold the parent pharmaceuticals and with regular format, the metabolites.

MS/MS conditions

Drying gas as well as nebulising gas was nitrogen generated from pressurized air in a N_2 nitrogen LC–MS (Claind, Teknokroma, Barcelona, Spain). The cone gas and the desolvation gas flows were set at 60 L/h and 1200 L/h, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) at 2×10^{-3} mbar in the T-Wave collision cell. Capillary voltages of −3.0 kV (negative ionization mode) and 3.5 kV (positive ionization mode) were applied. The interface temperature was set to 500 ºC and the source temperature to 120 ºC. Dwell time, inter-channel delay time, and inter-scan delay time were automatically assigned by the system software (MassLynx 4.1, Manchester, UK) using the auto-dwell feature.

MS and MS/MS optimization

Full-scan and MS/MS mass spectra were obtained from infusion of 1 mg/L individual standard solutions in methanol/water (50:50, v/v) at a flow rate of 10 μL/min.

All compounds were determined in positive ionization mode, with the exception of clofibric acid. Although this analyte might be analyzed in both positive and negative modes, the later was preferred because of the better sensitivity reached under this mode. All compounds showed an abundant $[M+H]^+$ ion (for clofibric acid $[M-H]^-$) which was selected as precursor ion.

For clopidogrel, clopidogrel carboxylic acid, losartan, losartan carboxylic acid and clofibric acid, the presence of one chlorine atom in their structure allowed the use of two different precursor ions (corresponding to ^{35}Cl and ^{37}Cl isotopes).

For clarithromycin, an additional sensitive transition was obtained selecting an in-source fragment by increasing the cone voltage. The fragmentation of this in-source fragment ion produced a highly abundant product ion, making possible the acquisition of other sensitive transition (see q_2 transition for the mentioned pharmaceutical in **Table 1**).

The method was divided in 24 overlapping retention time windows (one window per compound) and MRM data acquisition rates (dwell time, inter-channel delay time, inter-scan delay times) were automatically optimized.

3.2.3 Discusión de los resultados (artículo científico 7)

Optimización de las condiciones MS/MS

En primer lugar, se optimizaron los parámetros de masas para los iones precursores y producto de todos analitos seleccionados. Todos los compuestos se ionizaron en modo positivo, excepto el ácido clofíbrico para el que se escogió el modo de ionización negativo por su mayor sensibilidad en este modo de trabajo. Para este compuesto se seleccionó como ion precursor $[M-H]^-$ y para todos los restantes, $[M+H]^+$. En el caso de la claritromicina se seleccionó un fragmento como ion precursor en la segunda transición de confirmación, promoviendo su formación mediante la aplicación de un voltaje elevado. En aquellos compuestos con un átomo de cloro en su estructura fue posible escoger entre dos iones precursores distintos (^{35}Cl y ^{37}Cl).

Se seleccionaron tres transiciones por compuesto con el fin de asegurar de manera fiable su identificación. Tan sólo para uno de los analitos (N-acetil sulfametoxazol) se adquirieron dos transiciones debido a la presencia de tan sólo dos iones producto. El software utilizado en este trabajo había sido mejorado con respecto al empleado en los trabajos presentados en el Capítulo 2. Así, la nueva versión nos facilitó la optimización de los parámetros MS/MS mediante la selección automática de los mismos, por ejemplo, el *dwell time* adecuado para cada una de las ventanas de adquisición.

Optimización cromatográfica

Tras probar diversos disolventes (metanol y acetonitrilo) con distintos aditivos (HCOOH y NH_4Ac) a distintas concentraciones, se encontró que utilizando metanol con HCOOH (0.01%) la sensibilidad era mayor para la mayoría de los compuestos. Además, se probaron dos columnas de UHPLC (BEH y HSS T3), cuyas principales características se han mencionado en el Capítulo 2. En general, no se observaron diferencias en cuanto a la forma de pico; tan sólo para unos pocos compuestos sus picos cromatográficos eran un poco más estrechos utilizando la columna HSS T3. Además, utilizando esta columna

los compuestos estaban más retenidos, es decir, sus tiempos de retención eran ligeramente mayores, lo cual resultaba beneficioso para evitar la coelución de algunos compuestos y posibles efectos matriz derivados. Por estos motivos se seleccionó la columna HSS T3.

En el caso de los metabolitos, como se ha comentado en el apartado de Introducción de este artículo, por lo general se encuentran a bajos niveles de concentración. Por ese motivo se estudió la posibilidad de inyectar un elevado volumen de muestra con el fin de alcanzar bajos límites de detección para todos ellos. El sistema de inyección del equipo utilizado permite seleccionar distintos modos de inyección, en concreto, *partial loop*, *partial loop with needle overfill* y *full loop*. Brevemente, en los dos primeros casos el *loop* no se llena por completo (se puede inyectar hasta el 50% y el 75% del volumen de muestra del *loop* respectivamente). En la configuración *needle overfill* la exactitud, precisión y recuperación son mayores debido a que la muestra pasa a través de un dispositivo de detección del volumen; en contra, el tiempo de inyección de cada ciclo es mayor que el empleado en la primera configuración. En el modo *full loop* se inyecta el 100% del volumen de muestra del *loop*. De este modo, la precisión y la exactitud es mayor que en los otros dos casos. Por el contrario, su uso conlleva un mayor consumo de muestra y además podrían producirse problemas de la forma de pico de los compuestos más polares.

Se realizó una comparación de las tres configuraciones inyectando un patrón en solvente y un extracto de efluente (ambos preparados en metanol-agua, 10:90), utilizando un *loop* de 100 µL. Como era de esperar, la sensibilidad en el modo *full loop* era mayor, sin producirse un empeoramiento de la forma de pico, y por ello se seleccionó este modo de inyección.

Optimización de la etapa de extracción

Se estudió la eficiencia de extracción de tres cartuchos poliméricos: Oasis HLB (200 mg), Oasis MCX (150 mg) y Oasis MAX (150 mg). El primero resulta adecuado para una amplia gama de compuestos, tanto polares como apolares, mientras que los

cartuchos MCX están diseñados especialmente para la retención de compuestos con grupos básicos y los MAX resultan adecuados para compuestos ácidos. Los dos últimos cartuchos requieren típicamente un ajuste previo del pH de la muestra.

La elución de los cartuchos HLB se realizó con 8 mL de MeOH, en el caso de los MCX, con 4 mL de MeOH seguidos de 4 mL de MeOH 5% NH_4OH, y para los MAX se utilizaron 4 mL de MeOH seguidos de 4 mL de MeOH 5% HCOOH.

En general, el comportamiento de los tres cartuchos fue similar. Se escogió el cartucho HLB porque los valores de recuperación fueron más reproducibles (menor RSD). A continuación, se comparó la eficacia de extracción de los cartuchos HLB con diferente cantidad de sorbente (60 mg y 200 mg). Los resultados obtenidos fueron muy similares por lo que se decidió utilizar los cartuchos de 60 mg. De este modo, el volumen de solvente utilizado para la elución era menor (5 mL en lugar de 8 mL), gracias a lo cual se reducía el tiempo necesario para la evaporación del extracto y además el proceso resultaba ligeramente más económico.

Finalmente, se estudió la eficacia de extracción de este cartucho a pH 3, 7 y 9. En la siguiente gráfica se han representado los valores de recuperación de los compuestos a los diferentes pHs. No se han incluido los resultados correspondientes a dos de los fármacos estudiados (clopidogrel y losartán) porque en el momento de realizar estas pruebas todavía no disponíamos de su patrón. En general, el comportamiento de los compuestos fue similar por lo que se decidió no ajustar previamente el pH de la muestra, manteniéndolo próximo a la neutralidad. De esta manera el procedimiento resultaba más rápido y sencillo.

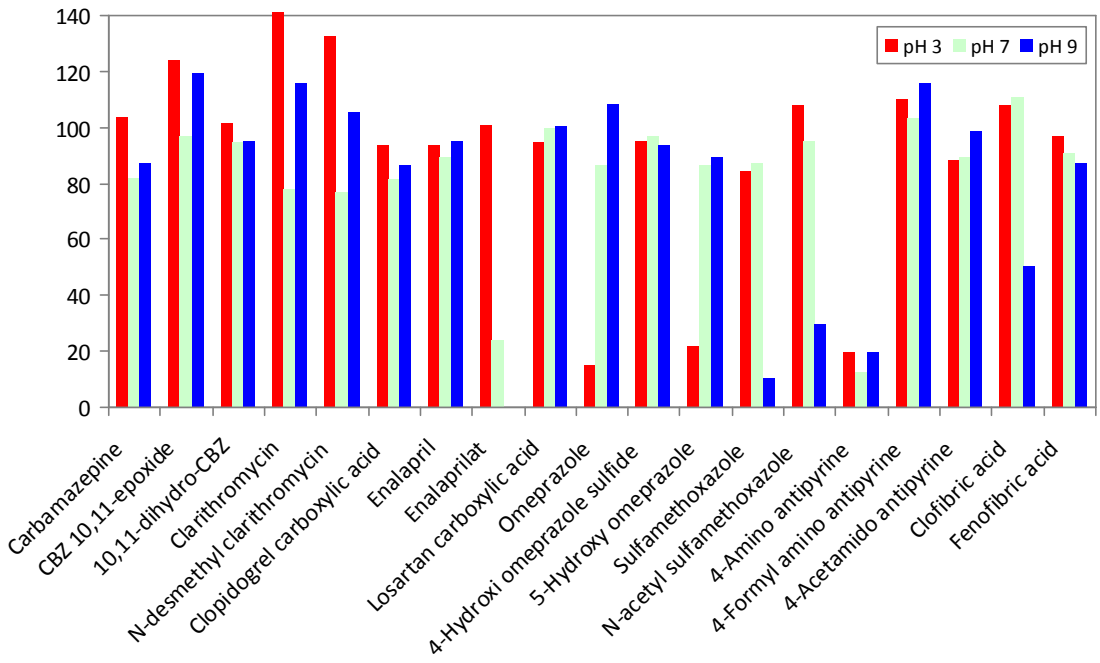

Figura 3.4 Recuperaciones obtenidas tras la extracción de los analitos con cartuchos Oasis HLB (60 mg) a diferentes valores de pH.

Validación del método

El método se validó en seis aguas superficiales a 0.02 µg/L y en seis efluentes, a dos niveles de concentración (0.1 y 0.4 µg/L). En las *Tablas S1 y S2 (artículo científico 7)* se muestran los valores de recuperación obtenidos en cada una de las doce aguas utilizadas, a los distintos niveles de fortificación. También se indica el valor promedio de recuperación para cada matriz a cada nivel junto con el valor de RSD para dar una visión global del proceso.

En agua superficial la validación fue satisfactoria para todos los compuestos excepto para cuatro de ellos. En el caso del enalaprilato se debió a su baja respuesta al nivel estudiado. Para los otros tres analitos, con el fin de conocer si los resultados fuera del rango establecido se debían a efecto matriz o a pérdidas durante el proceso de

extracción, para cada una de las aguas utilizadas en la validación se preparó, además de las muestras fortificadas antes de extracción por SPE, otra fortificada tras la etapa SPE. Este último eluato de SPE fortificado se comparó con patrones en solvente a la misma concentración con el fin de evaluar el efecto matriz. En la siguiente figura se ha representado el efecto matriz en cada una de las aguas objeto de estudio.

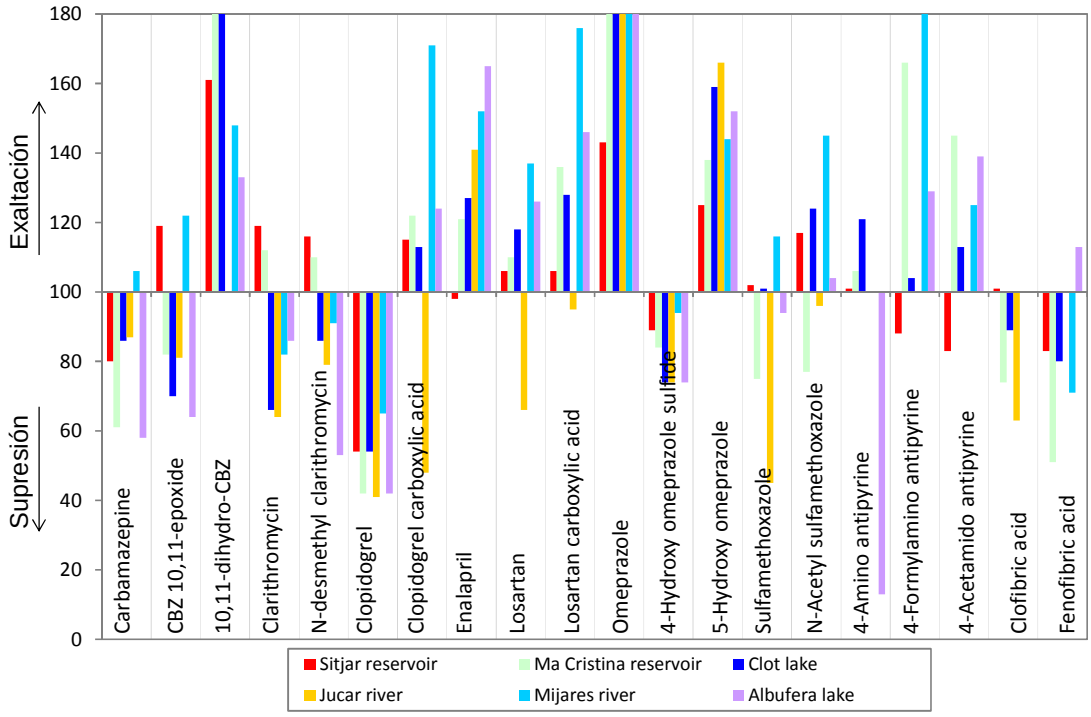

Figura 3.5 Efecto matriz en las seis aguas superficiales utilizadas en la validación.

Como puede observarse en la anterior figura, el efecto matriz de los analitos fue diverso: algunos compuestos sufrieron exaltación en todas las aguas analizadas (ej., 10,11-dihidro-10,11-dihidroxi carbamazepina, omeprazol, 5-hidroxi omeprazol), otros como 4-hidroxi omeprazol sulfuro, mostraron una ligera supresión de la señal para la totalidad de las muestras.

Aunque los resultados obtenidos parecen indicar que el comportamiento de los analitos fue muy similar en las seis aguas superficiales utilizadas, no siempre ocurrió de

ese modo. Por ejemplo, la claritromicina y su metabolito mostraron una supresión de la señal en cuatro de las aguas analizadas mientras que en las otras dos restantes se produjo una exaltación de la señal de ambos compuestos. Se observó una situación similar para el ácido fenofíbrico o para el compuesto 4-acetamidoantipirina. En este último caso, en todas las aguas excepto en una (concretamente, en la del embalse de Sitjar) se produjo exaltación de la señal.

Este estudio del efecto matriz nos permitió deducir, por ejemplo, que en el caso del clopidogrel las bajas recuperaciones se debieron al efecto matriz (supresión de la señal). Por el contrario, en el caso de 4-aminoantipirina, para el que se observó una cierta exaltación de la señal en la mayoría de las aguas superficiales estudiadas, los bajos valores de recuperación podían atribuirse a pérdidas durante el proceso de extracción.

En el agua de efluente, la experiencia adquirida en los trabajos anteriores permitía suponer que el efecto matriz sería más acusado que en el agua superficial y, por tanto, se requeriría algún tipo de corrección. En este trabajo disponíamos de tres compuestos marcados isotópicamente, número que resulta insuficiente para poder corregir el efecto matriz de todos los compuestos. Ante esta situación, se procedió a aplicar la dilución de las muestras con agua HPLC como un modo sencillo y eficiente para minimizar el efecto matriz. Se comprobó que una dilución x4 de las muestras resultó ser una solución rápida y sencilla para la gran mayoría de los analitos.

En la Figura 3.6 se ha representado el efecto matriz de cada una de las aguas de efluente. Si se comparara con la Figura 3.5 puede observarse que el efecto matriz es mucho más acusado que en las muestras superficiales, tal y como se preveía. De nuevo, se observó que aunque el comportamiento de los analitos fue muy similar en todas las aguas, puede existir una cierta variabilidad (ej. 4-formilaminoantipirina). A partir de los resultados obtenidos fue posible deducir que las elevadas recuperaciones de cinco de los compuestos estudiados (10,11-dihidro-10,11-dihidroxi carbamazepina, enalapril, 5-hidroxi omeprazol) se debieron a la exaltación de la señal producida por la matriz.

Por el contrario, la supresión de la señal experimentada por el clopidogrel, ácido clofíbrico y ácido fenofíbrico fue la causa de sus bajos valores de recuperación.

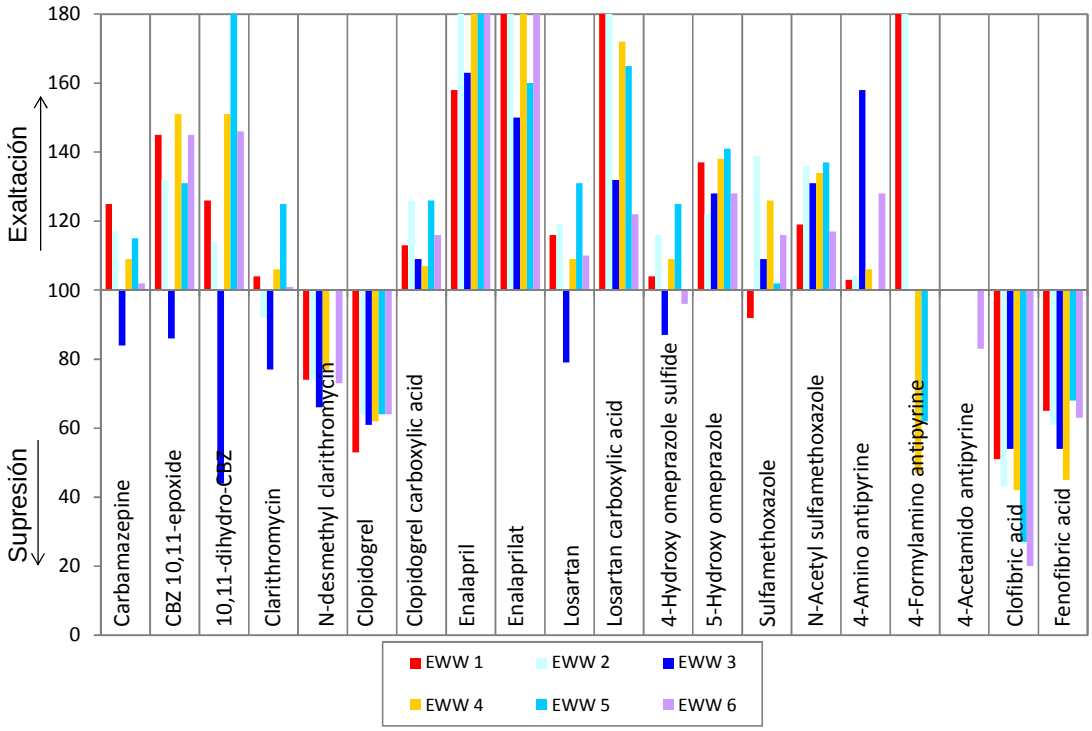

Figura 3.6 Efecto matriz en las seis aguas de efluentes utilizadas en la validación.

Uno de los compuestos (omeprazol) no pudo validarse en aguas de efluente a ninguno de los niveles ensayados debido probablemente a la baja estabilidad del patrón. Para comprobarlo se realizó un estudio de la estabilidad de este compuesto analizando una disolución recién preparada que se inyectó en el equipo cada siete días. Los resultados obtenidos indicaron que el omeprazol es un compuesto poco estable en disolución acuosa ya que se degrada a 4-hidroxi omeprazol sulfuro *(Figura 1, artículo científico 7*).

Una de las novedades que presenta este trabajo es el cálculo del LOQ para cada una de las muestras utilizadas en la validación, es decir, para cada analito disponíamos de seis valores de LOQ en agua superficial y otros seis en agua de efluente. Para cada

matriz se tomó el valor promedio como el valor final de LOQ, que es el valor que aparece en las *Tablas S1 y S2 (artículo científico 7)*. Se ha indicado también el rango de LOQ, es decir, el valor mínimo y máximo obtenido para este parámetro. En ambas matrices, pero especialmente en la de efluente, dicho rango es muy amplio. Este hecho que pone nuevamente de manifiesto que los analitos no están siempre afectados por el mismo efecto matriz y que incluso en un mismo tipo de agua pueden producirse variaciones importantes. Ante esto, los valores reportados para LOQ (y con mayor motivo para LOD) deberían tomarse como orientativos y con las debidas precauciones.

Análisis de muestras. Resultados en aguas superficiales y en efluentes

La metodología desarrollada se aplicó a muestras de agua superficiales y de efluentes.

En las muestras superficiales se detectaron todos los compuestos excepto tres metabolitos y un fármaco (omeprazol). Cabe mencionar que las concentraciones más elevadas correspondieron a dos de los metabolitos de la dipirona (0.9 μg/L) y a un metabolito de la carbamazepina (0.4 μg/L aproximadamente).

En relación a las muestras de efluente, cinco compuestos no se detectaron en ninguna ocasión, mientras que la frecuencia de detección del resto de compuestos fue muy elevada, hasta el punto de que 13 de ellos se detectaron en la totalidad de las muestras analizas.

Las concentraciones medianas de los metabolitos fueron similares a las de los compuestos de partida y en ocasiones fueron significativamente superiores (ej., los metabolitos de carbamazepina, clopidogrel y losartán).

Nuestros resultados indican que la presencia de metabolitos en las aguas es muy superior a la reportada hasta ahora, y que es necesario ampliar los listados de compuestos *target* incluyendo cada vez más metabolitos relevantes. Sólo de este modo,

se puede tener una visión global y más realista del posible impacto de los fármacos en el medio acuático.

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Capítulo 4

Conclusiones

Conclusiones

Del trabajo realizado en esta Tesis Doctoral, se pueden extraer las siguientes conclusiones generales:

1. La cromatografía líquida acoplada a espectrometría de masas con analizador de triple cuadrupolo es una técnica muy valiosa para la determinación (cuantificación y confirmación) de fármacos y de metabolitos/productos de transformación en diferentes tipos de aguas.

2. La aplicación de una etapa de preconcentración, basada en la extracción en fase sólida, permite alcanzar la sensibilidad necesaria para detectar los bajos niveles de concentración de los fármacos, especialmente en las muestras de efluente urbano y agua superficial, generalmente en el orden de sub-ppb.

3. La cromatografía líquida de ultra presión (UHPLC) es una poderosa técnica para el desarrollo de métodos rápidos y sensibles cuando se acopla con espectrometría de masas. Proporciona alta resolución y sensibilidad así como velocidad de análisis.

4. La resolución mejorada proporcionada por UHPLC solamente se puede aprovechar realmente cuando se acopla con un espectrómetro de masas con elevada velocidad de barrido. Gracias a ello también es posible adquirir más transiciones SRM por compuesto en una misma inyección, aumentando la confianza en la identificación de los analitos.

5. El desarrollo y aplicación de métodos multirresiduales en los que se incluye un elevado número de fármacos de diversas familias requiere un compromiso en cuanto a la selección de las condiciones experimentales más adecuadas: separación cromatográfica, detección MS/MS y tratamiento de la muestra. La corrección del efecto matriz ha resultado ser la fase más complicada cuando se analizan diversos tipos de aguas.

Este efecto es mucho más severo en el caso de matrices complejas (influente y efluente) y su corrección cuando se trata de métodos multirresiduales resulta complicada. El uso de analitos marcados isotópicamente como patrones internos y la dilución de las muestras han sido las estrategias utilizadas para corregir/minimizar el efecto matriz en esta Tesis.

6. El uso del propio analito marcado isotópicamente como patrón interno ha permitido la corrección del efecto matriz en todas las muestras ensayadas. Cuando el compuesto marcado no está disponible, el empleo de otros marcados distintos (marcados análogos) puede resultar una buena alternativa, aunque no siempre asegura una corrección satisfactoria para todas las aguas analizadas, por lo que su uso debe comprobarse cuidadosamente y en un número notable de muestras de diferente composición y origen.

7. La validación de los métodos analíticos en diferentes muestras de agua ha proporcionado una visión más realista sobre el comportamiento y robustez del método en situaciones reales en las que deben analizarse distintas muestras, ya que la composición de las aguas es muy variable, aún siendo todas del mismo tipo (ej. superficiales).

8. Los analgésicos/antiinflamatorios y los reguladores lipídicos son los grupos de fármacos que se han detectado con más frecuencia en las aguas residuales y superficiales. Entre los antibióticos, destacan las quinolonas y los macrólidos. Por compuestos, los más detectados han sido el diclofenaco, 4-aminoantipirina, ketoprofeno, naproxeno, gemfibrozil y venlafaxina.

9. El comportamiento de los fármacos frente al proceso de depuración de una EDAR es muy diverso. Para la mayoría de los compuestos, la eficacia de eliminación utilizando un tratamiento convencional de depuración (tratamiento primario y secundario) es satisfactoria, aunque sólo para

unos pocos la eliminación es completa. Por ejemplo, acetaminofeno y enalapril se eliminaron completamente, mientras que para algunos fármacos utilizados para el tratamiento del colesterol, como atorvastatina y pravastatina, su eliminación fue parcial. Finalmente, en el caso de los ansiolíticos y de algunos antibióticos, su eliminación fue prácticamente nula.

10. El acoplamiento UHPLC-QTOF MS se ha mostrado como una herramienta analítica muy valiosa para la detección e identificación de metabolitos/TPs de fármacos en muestras medioambientales.

11. El análisis retrospectivo permite detectar e identificar cualquier compuesto de interés en cualquier momento posterior a los análisis iniciales sin realizar ningún análisis adicional, siempre y cuando las condiciones de tratamiento de muestra, separación cromatográfica e ionización resulten adecuadas para los compuestos que se investigan. En este trabajo se han podido detectar de este modo cuatro metabolitos, concretamente, N-desmetil claritromicina, 14-hidroxi-claritomicina, ácido fenofíbrico y ácido carboxílico de clopidogrel, en muestras que ya habían sido analizadas por LC-QTOF MS.

12. Se ha demostrado la utilidad de la espectrometría de masas de alta resolución cuando se investigan metabolitos procedentes de un mismo fármaco y que comparten las mismas transiciones. En este tipo de situaciones la separación cromatográfica resulta esencial para evitar falsos positivos (falsas identificaciones). El uso de QTOF MS ha permitido identificar dos metabolitos de la dipirona (4-FAA y 4-AAA) que comparten las mismas transiciones que otro de los metabolitos (4-AA) y que pueden dar lugar, por tanto, a falsos positivos de 4-AA si los picos cromatográficos no están bien resueltos.

13. Los resultados obtenidos en los análisis realizados por QTOF y QqQ demuestran que los metabolitos de los fármacos están presentes en el

agua, incluso con mayor frecuencia y a niveles de concentración más altos que los fármacos de partida. Por este motivo, se requiere ampliar la investigación sobre estos compuestos y desarrollar más metodología analítica que la que existe actualmente.

Chapter 4

Conclusions

Conclusions

As a result of the research performed in this Thesis, several general conclusions can be extracted:

1. Liquid chromatography coupled to mass spectrometry with triple quadrupole mass analyzer is a powerful analytical tool for the determination (quantification and confirmation) of pharmaceuticals and metabolites/transformation products in different types of water.

2. The application of a pre-concentration step, based on solid-phase extraction, allows to reach the sensitivity required to detect the low pharmaceutical concentrations (usually at the sub-ppb levels), especially in urban wastewater and surface water samples.

3. Ultra-high performance liquid chromatography (UHPLC) is a powerful technique that facilitates the development of fast and sensitive methods when coupled to mass spectrometry. It provides high resolution and sensitivity (narrow peaks) as well as elevated speed of analysis.

4. The improved resolution offered by UHPLC is only fully achievable when coupled to fast-acquisition mass spectrometers. This also makes possible acquiring more SRM transitions per compound in the same injection, increasing the confidence in the identification of analytes.

5. The development of multi-residue/multi-class methods requires a compromise in the selection of experimental conditions: chromatographic separation, MS/MS detection and sample treatment. Matrix effect correction has turned out the most complicated step when different types of water are analyzed. This effect is more severe in complex matrices (influent and effluent wastewater) and its correction when dealing with multi-residue methods is very complicated. The use of isotope-labelled compounds as internal standards and sample

dilution have been the strategies used to correct/minimize matrix effect in this Thesis.

6. The use of the analyte isotope-labelled compound as internal standard has allowed the satisfactory matrix effect correction in all the samples tested. When the analyte ILIS is not available, the use of another compound (analogue isotope-labelled internal standard) may be an adequate alternative, although the satisfactory correction for all the samples analyzed cannot be ensured. Thus the use of analogues ILIS should be checked carefully in a notable number of samples.

7. Validation of analytical methods in different water samples has provided a more realistic overview of the method performance and its robustness in real situations where different samples must be analyzed, as the composition of aqueous samples is never the same, even when analyzing the same sample type (eg. surface water).

8. Analgesics/anti-inflammatories and lipid regulators are the more frequently groups found in wastewater and surface waters. Among antibiotics, quinolones and macrolides were the most detected. Diclofenac, 4-aminoantipyrine, ketoprofen, naproxen, gemfibrozil and venlafaxine are the most frequently detected pharmaceuticals.

9. The behaviour of pharmaceuticals in the treatment process of a WWTP is very diverse. For the most of the compounds, the removal efficiency of conventional treatment process (primary and secondary treatment) is satisfactory, although only for a few compounds the elimination is complete. For example, acetaminophen and enalapril were completely removed, while for some pharmaceuticals used for cholesterol treatment, as atorvastatin and pravastatin, the removal was only partial. Finally, in the case of anxiolytics and some antibiotics their removal was non-existent.

10. UHPLC coupled to QTOF MS has be shown to be a very valuable analytical tool for the detection and identification of pharmaceutical metabolites/TPs in environmental samples.

11. The retrospective analysis in a QTOF MS allows the identification of any compound of interest at any time without the need for additional analysis, provided that sample treatment conditions, chromatographic separation and ionization mode are suitable for the compounds investigated. In this work, retrospective analysis has allowed the identification of four metabolites, such as N-desmethyl clarithromycin, 14-hydroxy-clarithromycin, fenofibric acid and clopidogrel carboxylic acid in water samples previously analyzed by LC-QTOF MS.

12. The usefulness of high resolution mass spectrometry for investigation of metabolites derived from the same pharmaceutical that may share the same transitions has been demonstrated. Under these circumstances, the appropriate chromatographic separation is crucial for avoiding false positives (uncorrect identifications). The use of QTOFMS has allowed identifying two dipyrone metabolites (4-FAA y 4-AAA) which share the same transitions than another of its metabolites (4-AA). This situation can lead to report false positives of 4-AA if the chromatographic peaks are not well resolved.

13. The results obtained in the analysis by QTOF and QqQ demonstrate that pharmaceutical metabolites can be present in waters, even at higher frequency and concentrations than the parent compounds. Thus, more attention towards these compounds should be paid in the near future, and appropriate analytical methodology needs to be developed to efficiently monitoring pharmaceutical metabolites in the aquatic environment.

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