INVESTIGACIÓN DE FÁRMACOS Y DROGAS DE ABUSO EN EL ÁMBITO DE LA SALUD PÚBLICA.

IDENTIFICACIÓN DE PRODUCTOS DE TRANSFORMACIÓN/METABOLITOS EN EL MEDIO AMBIENTE ACUÁTICO.

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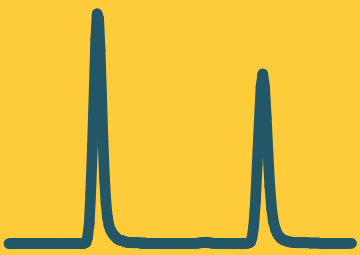

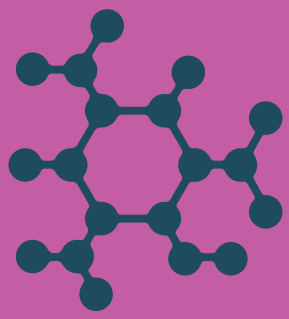

UNIVERSITAT JAUME·I

CLARA BOIX SALES

TESIS DOCTORAL

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Instituto Universitario de Plaguicidas y Aguas

INVESTIGACIÓN DE FÁRMACOS Y DROGAS DE ABUSO EN EL ÁMBITO DE LA SALUD PÚBLICA.
Identificación de productos de transformación/metabolitos en el medio ambiente acuático.

Tesis Doctoral

CLARA BOIX SALES

2014

El **Dr. Félix Hernández Hernández**, Catedrático de Química Analítica, y la **Dra. María Ibáñez Martínez,** Investigador contratado Doctor en Química Analítica, de la Universitat Jaume I de Castellón,

Certifican: que la Tesis Doctoral "Investigación de fármacos y drogas de abuso en el ámbito de la salud pública. Identificación de productos de transformación/metabolitos en el medio ambiente acuático" 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 **Clara Boix Sales**.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 29 de julio de 2014.

Fdo. Dr. Félix Hernández Hernández Fdo. Dra. María Ibáñez Martínez

Este trabajo se ha realizado mediante la concesión de una ayuda para el desarrollo de acciones científicas del programa de investigación de excelencia Prometeo 2009 (2009/054) de la Generalidad Valenciana, desde el 1 de octubre de 2010.

Clara Boix Sales realizó una estancia en el ***Institute for Biodiversity and Ecosystems Dynamics*** **(IBED)** en la Universidad de Ámsterdam (Países Bajos), desde el 1 de noviembre al 21 de diciembre de 2012. La estancia estuvo supervisada por el Dr. John Parsons, quien dirigió los experimentos de biodegradación utilizando lodos activados. De dicha estancia surgió el trabajo reportado en el Artículo Científico 6 "*Biodegradation of pharmaceuticals in surface water and during waste water treatment: identification and occurrence of transformation products*". Adicionalmente, disfruto de una estancia en el ***Istituto di Ricerche Farmacologiche Mario Negri*** de Milán (Italia), desde el 17 de febrero al 28 de marzo de 2014. La estancia en este centro de investigación, supervisada por el Dr. Ettore Zuccato, permitió demostrar la utilidad de los estudios previamente desarrollados, en un estudio aplicado realizado en Italia, con otras aguas y con otro equipo instrumental diferente. De dicha estancia surgió el trabajo reportado en el Artículo Científico 4 *"Investigation of omeprazole and venlafaxine metabolites in wastewater making use of high resolution mass spectrometry"*.

Los trabajos realizados en la presente memoria han sido en parte financiados por proyectos del Ministerio de Ciencia y Tecnología de la Generalidad Valenciana y del Collaborative Research on Environment and Food Safety (ISIC/2012/2016).

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. Alexander L.N. Van Nuijs (Toxicological Center University of Antwerp Universiteitsplein, Belgium).

A la “ueli”

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durant aquests dos últims mesos han suportat de primera mà el meu estrès, gràcies per fer-me riure en les vostres "parides".

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Un trosset d'aquesta Tesi és també vostra!

Clara

Resumen

Uno de los aspectos más importantes y que está suscitando mayor preocupación en el ámbito de la salud pública es el control de contaminantes orgánicos emergentes en el medio ambiente acuático, ya que puede afectar de forma notable a su calidad y a los organismos vivos que habitan en él. Los fármacos, por su elevado consumo, y las drogas de abuso, por su peligrosidad e ilegalidad, son los compuestos que están despertando mayor interés. Dichos compuestos son consumidos por los seres humanos, y los fármacos en menor medida por animales, siendo posteriormente eliminados de nuestro organismo como compuesto intacto o en forma de metabolitos. Todos estos compuestos llegan a las estaciones depuradoras pudiendo alcanzar las aguas superficiales si no son eficientemente eliminados tras los tratamientos aplicados a las aguas residuales. Por otra parte, aun siendo parcial o totalmente eliminados, pueden generar productos de transformación (TPs), los cuales también pueden llegar al medioambiente. En algunos casos, los TPs son desconocidos, por lo que la falta de información sobre sus posibles efectos sobre nuestro ecosistema es prácticamente total. La escasa información reportada sobre presencia y efectos de TPs y metabolitos de contaminantes emergentes en el medio ambiente es, en parte, debida a la falta de metodología analítica para su determinación.

La presente Tesis Doctoral se ha centrado en el estudio del potencial y aplicaciones analíticas del acoplamiento instrumental Cromatografía Líquida de Ultra-alta Resolución (UHPLC) - Espectrometría de Masas (MS) con analizadores de triple cuadrupolo (QqQ) y cuadrupolo-tiempo de vuelo (QTOF) para la determinación de fármacos y drogas de abuso, principalmente en aguas residuales y superficiales, con especial énfasis en la investigación de sus metabolitos y productos de transformación.

El trabajo realizado se divide en 5 partes bien diferenciadas:

En la primera parte se ha estudiado el potencial del acoplamiento UHPLC-MS/MS para la determinación cuantitativa de 40 contaminantes emergentes (5 drogas de abuso y 35 fármacos frecuentemente consumidos) en aguas. El método desarrollado se basa en la inyección directa de la muestra de agua en el sistema cromatográfico y la posterior medida usando un analizador de masas de triple cuadrupolo de última generación. El método fue optimizado y validado tanto en agua superficial como en efluente urbano. La aplicabilidad del método se demostró analizando aguas de diferentes tipos y orígenes, llegando a detectar hasta 32 compuestos entre los 40 seleccionados, entre los que cabe destacar por su mayor frecuencia la carbamazepina (100%), el valsartán (90%), el levamisol, la trimetoprima, el sulfametoxazol o la venlafaxina (80%). Los compuestos detectados a mayor concentración, tanto en aguas residuales urbanas como medioambientales fueron los metabolitos de la dipirona.

En la segunda parte se ha llevado a cabo un estudio sobre el fármaco omeprazol, con el objetivo de investigar las razones de su escasa detección en aguas pese a ser uno de los medicamentos más consumidos en todo el mundo. Se llevaron a cabo experimentos de degradación en el laboratorio, tratando de reproducir algunos tratamientos aplicados en las estaciones depuradoras (por ejemplo, cloración o radiación ultravioleta) y las posibles reacciones de degradación producidas en el medio ambiente (por ejemplo, foto-degradación). El análisis de las muestras sometidas a degradación mediante UHPLC-QTOF en modo MS^E y el procesamiento de los datos utilizando el software MetaboLynx XS™, permitió detectar y elucidar 17 TPs resultantes de procesos de hidrólisis, cloración y foto-degradación.

Adicionalmente, se realizó un estudio sobre el metabolismo del omeprazol en humanos. Para ello, se investigaron las orinas de tres voluntarios tras la ingesta de

omeprazol, tratando de detectar nuevos y abundantes metabolitos del fármaco. Las orinas se analizaron por UHPLC-QTOF (MS^E) identificando hasta 24 metabolitos, comunes en los tres voluntarios.

Posteriormente, con el fin de comprobar la utilidad de los trabajos de metabolismo y degradación realizados, se analizaron 52 muestras de aguas recogidas en el entorno de la Comunidad Valenciana (25 residuales y 27 superficiales) mediante LC-MS/MS QqQ y LC-QTOF MS con el objetivo de detectar los metabolitos y TPs identificados en los experimentos de laboratorio. Ello permitió detectar 4 TPs minoritarios y hasta 14 metabolitos, mientras que el omeprazol no estuvo presente en ninguna de las muestras.

Finalmente, con el objetivo de comprobar la presencia de los mencionados metabolitos de omeprazol en aguas italianas, se analizaron 30 muestras de influente procedentes de diez ciudades distintas mediante HPLC-Orbitrap MS. Los resultados obtenidos en las muestras españolas e italianas fueron semejantes, detectando los metabolitos en ambos países. Cabe destacar que algunos de los compuestos detectados en la aguas y reportados en esta Tesis Doctoral eran desconocidos hasta ahora.

La tercera parte se ha centrado en la investigación del principal metabolito del cannabis (THC-COOH) en muestras acuáticas. Se realizaron experimentos de degradación similares a los de la segunda parte. Tras el análisis mediante UHPLC-QTOF MS, se detectaron e identificaron tentativamente 19 TPs del THC-COOH. Con el fin de investigar la presencia de dichos TPs en aguas residuales y superficiales, se desarrolló un método analítico por LC-QqQ MS, usando la información obtenida del QTOF MS para seleccionar los iones en modo *Selected Reaction Monitoring* (SRM). Tras su aplicación a muestras de aguas residuales y superficiales, se detectaron varios de los TPs identificados en el laboratorio, entre los que cabe destacar pos su

mayor frecuencia de detección los formados en las experiencias de de foto-degradación por simulación de la luz solar.

En la cuarta parte se ha llevado a cabo un estudio de biodegradación con cinco fármacos de consumo frecuente (irbesartán, venlafaxina, ofloxacino, ibuprofeno y gemfibrozil), sometidos a experimentos con lodos activados y con aguas superficiales, tratando de investigar cómo actúan los microorganismos sobre ellos. Tras los experimentos de laboratorio, análisis mediante UHPLC-QTOF MS y procesamiento de los datos, se pudieron detectar 8 TPs de irbesartán, 6 de venlafaxina, 1 de ofloxacino, 6 de ibuprofeno y 1 de gemfibrozil. Estos TPs, junto con los cinco fármacos de partida, se buscaron retrospectivamente en muestras de agua de efluente y superficial, que ya habían sido analizadas por LC-QTOF MS. Se pudo confirmar la presencia de diversos TPs, algunos de los cuales fueron detectados en un mayor número de muestras que sus respectivos fármacos inalterados.

Finalmente, en la quinta parte se ha explorado el potencial del LC-QTOF MS para la investigación de fármacos, pero en este caso sobre otro tipo de muestras diferente y en el ámbito de la seguridad alimentaria. Se desarrolló una metodología de *screening* rápido de tipo cualitativo de 116 fármacos de uso humano y animal en muestras de pienso para el ganado. La metodología de *screening* fue validada cualitativamente (detección e identificación) y aplicada a cinco matrices de pienso animal: caprino, bovino, cunícola, avícola y porcino. Tras el análisis de 22 muestras de piensos se pudieron detectar 11 fármacos; entre ellos, cabe mencionar los prohibidos α y β-nandrolona en pienso bovino, cunícola, caprino y/o porcino. También se exploró el potencial cuantitativo de la técnica LC-QTOF MS para los compuestos que se detectaron en las muestras.

Summary

The control of emerging compounds in aquatic environment is a subject of present concern and one of the most important fields of research in analytical chemistry. The presence of these compounds may affect the water quality as well as the organisms living there. Pharmaceuticals, due to their wide use, and drugs of abuse, due to their consumption, dangerousness and illegality, have become one of the most relevant compounds. These compounds are mainly consumed by humans, and some pharmaceuticals also by animals, being then excreted as unchanged compound or as metabolites. The wide majority of these compounds reach the wastewater treatment plants (WWTPs), and also surface waters if they are not efficiently eliminated by the treatments applied in WWTPs. When these drugs are partially or completely eliminated/degraded, they may generate transformation products (TPs), which might also reach the environment. In some cases, TPs are unknown compounds, so the information about their possible presence and effects in our ecosystem is almost missing. One of the reasons of the scarce information reported about the presence and effects of TPs/metabolites of emerging contaminants in the environment is the lack of suitable analytical methodology for their determination.

The present Doctoral Thesis has focused on the study of the analytical potential and applications of Ultra-High Performance Liquid Chromatography (UHPLC) coupled to mass spectrometry using triple quadrupole (QqQ) and hybrid quadrupole time-of-flight (QTOF) for the determination of pharmaceuticals and drugs of abuse in water. The work has been directed mainly towards effluent wastewater (EWW) and surface water (SW), emphasizing the investigation on their metabolites and transformation products with the help of laboratory degradation experiments.

The work has been divided into five parts:

In the first part, the potential of UHPLC-MS/MS has been studied for the quantitative determination of 40 emerging contaminants (5 drugs of abuse and 35 pharmaceuticals highly consumed). The developed method is based on direct water sample injection on a last-generation triple quadrupole. The method was validated in surface water as well as in effluent wastewater samples. The applicability of the method was illustrated analyzing samples from different origin and type, detecting 32 out of the 40 selected compounds. Carbamazepine (100%), valsartan (90%), levamisole, trimethoprim, sulfamethoxazole and venlafaxine (80%) were the most frequently detected. It is important to highlight the presence of dipyrone metabolites, as they appeared at higher concentrations.

In the second part of the Thesis, a deep study on the pharmaceutical omeprazole is carried out in order to investigate why it is scarcely present in waters despite being one of the most consumed drugs around the world. Laboratory controlled degradation experiments were performed trying to simulate some treatments usually applied in the wastewater treatment plants (i.e. chlorination or ultraviolet photo-degradation (UV)) as well as the possible degradation reactions which occur in the environment (i.e. photo-degradation). The degraded samples were analyzed by UHPLC-QTOF MS acquiring in MS^E, with the result of 17 TPs found from hydrolysis, chlorination and photo-degradation experiments that were tentatively elucidated.

Additionally, a human metabolism study on omeprazole was performed after the administration of omeprazole to volunteers, in order to detect new and abundant omeprazole metabolites. Urine samples were analyzed by LC-QTOF MS (MS^E), yielding 24 common metabolites for three volunteers.

The usefulness of metabolism and degradation experiments previously performed was tested by analysis of 52 water samples collected around the Valencian Region (25 wastewater and 27 surface waters) using LC-MS/MS QqQ and LC-QTOF MS, with the objective to detect metabolites and TPs discovered in laboratory degradation experiments. This allowed detecting 4 minor TPs and up to 14 metabolites, while omeprazole was not present in any of the samples.

Finally, with the purpose to support the results obtained in our laboratory, the presence of the omeprazole metabolites was investigated in Italian waters, by analyzing 30 influent wastewater (IWW) samples from ten different cities using HPLC-Orbitrap MS. The results obtained in Spanish and Italian samples were similar, detecting the same metabolites in both countries. Interestingly, some compounds detected in waters and discovered in this Doctoral Thesis had not been reported until now.

The third part deals with the investigation of the main cannabis metabolite (THC-COOH) in aquatic samples. Degradation experiments (similar to the second part) were carried out in the laboratory. The use of UHPLC-QTOF MS allowed the detection and tentative identification of 19 TPs of THC-COOH. In order to investigate the presence of these TPs in wastewaters and surface waters, an analytical method was developed based on LC-MS/MS QqQ using the information obtained from QTOF MS for selecting the SRM (selected reaction monitoring) transitions. After analysis of several wastewater and surface water samples, some TPs were detected. It is important to notice that TPs generated in sunlight photo-degradation experiments were detected in a high number of samples.

In the fourth part, a biodegradation study on five pharmaceuticals (irbesartan, venlafaxine, ofloxacin, ibuprofen and gemfibrozil) has been carried out in experiments with activated sludge and surface waters, trying to investigate the

action of microorganisms. After laboratory experiments, analysis by LC-QTOF MS and data processing, 8 TPs for irbesartan, 6 for venlafaxine, 1 for ofloxacin, 6 for ibuprofen and 1 for gemfibrozil were found. All these TPs, together with the five parent pharmaceuticals, were retrospectively searched in effluent and surface waters. The presence of several TPs could be confirmed in the samples. Some of them were found in a number of samples higher than their respective parent compounds.

Finally, in the fifth part, the potential of the LC-QTOF MS has been explored for the investigation of pharmaceuticals, but moving to the food safety field. A rapid screening methodology was developed for 116 human and veterinary pharmaceuticals in animal feed samples. The screening methodology was qualitatively (detection and identification) validated and applied to five animal feed matrices: goat, bovine, rabbit, poultry and pork. Later, 22 feed samples were analyzed with the result of detecting up to 11 drugs, among them, the prohibited α and β-nandrolone, in bovine, rabbit, goat and/or pork feeds.

ÍNDICE GENERAL

CAPÍTULO 2.

Desarrollo de metodología analítica multi-residual basada en LC-MS/MS para la determinación de fármacos y drogas de abuso en aguas

CAPÍTULO 3.

Investigación de la presencia de omeprazol en aguas: estudio de degradación y metabolismo

CAPÍTULO 7.

CAPÍTULO 8.

ÍNDICE ACRÓNIMOS

AA	AminoAntipyrine
AAA	Acetyl AminoAntipyrine
APCI	Atmospheric Pressure Chemical Ionization interface
APIs	Atmospheric Pressure Interfaces
ATC	Anatomic, Therapeutic, Chemical classification system
BEH	Ethylene Bridged Hybrid
BOD	Biochemical Oxygen Demand
CID	Collision Induced Dissociation
C-Trap	C-shaped ion Trap analyzer
D	Deuterium
DMA	Directiva del Marco del Agua
EDARs	Estaciones Depuradoras de Agua Residual
EMA	European Medicines Agency
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EQSD	Environmental Quality Standards Directive
ESI	ElectroSpray Interface
EU	European Union
EWW	Effluent WasteWater
FAA	Formyl AminoAntipyrine
FDA	Food and Drug Administration U.S.

FT	Fourier Transform
FWHM	Full-Width Half Maximum
GC	Gas Chromatography
HCOOH	Formic Acid
HE	High Energy
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IBED	Institute for Biodiversity and Ecosystems Dynamics
ICR	Ion Cyclotron Resonance
IS	Internal Standard
IT, LIT	Linear Ion Trap
IUPA	Instituto Universitario de Plaguicidas y Aguas
IWW	Influent WasteWater
KWR	Watercycle Research Institute
LC	Liquid Chromatography
LC-MS	Liquid Chromatography coupled Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled tandem Mass Spectrometry
LE	Low Energy
LOQ	Limit Of Quantification
LOI	Limit Of Identification
LRMS	Low Resolution Mass Spectrometry

MDMA	3,4-metilendioximetanfetamina
ME	Matrix Effect
MRL	Maximum Residue Level
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	Mass/charge ratio
NH_4Ac	Ammonium acetate
nw-XICs	narrow width-eXtracted Ion Cromatograms
OMs	Omeprazole Metabolites
OTPs	Omeprazole Transformation Products
ppb	Part Per Billion
ppm	Part Per Million
ppt	Part Per Trillion
Q	Quadrupole analyzer
q/Q	Ion ratio
QqQ	Triple Quadrupole analyzer
QTOF	hybrid Quadrupole Time Of Flight analyzer
RSD	Relative Standard Deviation
Rt	Retention time
SDL	Screening Detection Limit
S/N	Signal/Noise ratio

SPE	Solid Phase Extraction
SRM	Selected Reaction Monitoring
SW	Surface Water
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol
TIC	Total Ion Current
TOF	Time Of Flight analyzer
TPs	Transformation Products
UHPLC	Ultra-High Performance (or Pressure) Liquid Chromatography
UNODC	United Nations Office on Drugs and Crime
UV	UltraViolet
WW	WasteWater
XIC	eXtracted Ion Cromatogram

ÍNDICE FIGURAS

ÍNDICE TABLAS

OBJETIVOS, METODOLOGÍA Y PLAN DE TRABAJO

Objetivos

El **principal objetivo** de la presente Tesis Doctoral es investigar las capacidades analíticas de la cromatografía líquida de ultra resolución (UHPLC) acoplada a espectrometría de masas en tándem (MS/MS) con analizadores de triple cuadrupolo (QqQ), híbrido cuadrupolo-tiempo de vuelo (QTOF) y LTQ Orbitrap, para la determinación de fármacos, drogas de abuso y sus metabolitos/productos de transformación en aguas y piensos, tratando de avanzar en la problemática ambiental y en aspectos de interés en seguridad alimentaria.

Por un lado, se pretende desarrollar, validar y aplicar metodología analítica avanzada, basada en UHPLC-MS/MS QqQ, para la determinación cuantitativa de compuestos emergentes (fármacos y drogas de abuso) de elevado consumo en aguas de distinta naturaleza.

Por otro lado, tras aplicar la metodología anteriormente mencionada y, como consecuencia de la escasa o nula detección de determinados compuestos altamente consumidos, se plantea el objetivo de investigar la posible presencia de metabolitos y productos de transformación (TPs) más relevantes en aguas mediante LC-QTOF MS y LC-MS/MS QqQ.

Finalmente, se plantea estudiar el potencial de la LC-QTOF MS para el *screening* y evaluación semi-cuantitativa de fármacos de uso humano y animal en muestras de pienso animal.

Con el fin de alcanzar el objetivo principal, se establecieron los **objetivos específicos** que se indican a continuación:

1. Desarrollar metodología analítica avanzada para la determinación multi-residual de fármacos y drogas de abuso ampliamente utilizados, basada en

la inyección directa de la muestra de agua al sistema UHPLC-MS/MS con analizador de triple cuadrupolo de última generación.

2. Aplicar la metodología analítica descrita a muestras de agua residual de efluente urbano y superficial, procedentes de distintos puntos de la Comunidad Valenciana. Comparar los resultados con un método de extracción en fase sólida (SPE) *on-line* seguido de LC-MS/MS QqQ, desarrollado por un laboratorio acreditado con el que colabora asiduamente nuestro grupo de investigación.
3. Estudiar la degradación de fármacos y drogas seleccionados en condiciones controladas de laboratorio, con el fin de simular procesos de hidrólisis y radiación solar, que ocurren típicamente en el medio ambiente, e identificar los principales productos de transformación.
4. Estudiar la degradación de fármacos y drogas seleccionados reproduciendo los tratamientos de desinfección utilizados en las estaciones de aguas residuales (EDARs): hidrólisis, biodegradación, cloración y foto-degradación ultravioleta.
5. Desarrollar metodología analítica basada en LC-QTOF MS para la identificación y elucidación estructural de TPs generados tras los experimentos de degradación llevados a cabo en el laboratorio.
6. Aplicar la metodología propuesta a compuestos emergentes de elevado consumo, algunos de ellos escasamente detectados en las aguas, tales como omeprazol, venlafaxina, ibuprofeno, gemfibrozil, ofloxacino, irbesartán, así como el principal metabolito del cannabis THC-COOH.

7. Investigar el metabolismo del omeprazol en orina de tres voluntarios mediante UHPLC-QTOF MS, con el fin de detectar, identificar y elucidar metabolitos relevantes no descritos en bibliografía.
8. Realizar análisis retrospectivo en muestras de agua ya analizadas mediante UHPLC-QTOF MS, con el objetivo de investigar la presencia de los TPs y metabolitos identificados previamente en los ensayos a nivel de laboratorio.
9. Utilizar la información obtenida mediante UHPLC-QTOF MS sobre la molécula (des)protonada e iones fragmento para desarrollar métodos analíticos basados en LC-MS/MS (QqQ) que permitan la determinación, a nivel de trazas, de metabolitos/TPs en muestras de agua.
10. Investigar la presencia de metabolitos/TPs, utilizando la aproximación basada en la búsqueda de iones fragmento comunes, en aguas residuales y superficiales mediante LC-QTOF MS.
11. Poner a punto un método analítico de *screening* basado en UHPLC-QTOF MS para 116 fármacos de uso humano y animal en muestras de pienso, y aplicarlo a distintas matrices de pienso animal, como caprino, bovino, cunícola, avícola y porcino.

Metodología y plan de trabajo

La metodología de trabajo seguida para la puesta a punto de los métodos analíticos que conforman la presente Tesis Doctoral se puede dividir según se trate de métodos cuantitativos o cualitativos:

Metodología seguida en **métodos cuantitativos**:

1. Revisión bibliográfica y selección de los posibles analitos según el consumo reportado, la peligrosidad/toxicidad y la potencial presencia en el medio ambiente.

2. Estudio de las condiciones óptimas de MS y MS/MS mediante la inyección de patrones de referencia.

   a. Adquisición en modo "barrido" (*scan*) para establecer el modo de ionización y la energía de cono óptima de cada analito.
   b. Adquisición en modo "barrido de iones producto", aislando el ion precursor, con el fin de optimizar la energía de colisión y obtener los iones producto característicos.
   c. Selección de 3 transiciones (MS/MS) por compuesto para facilitar su determinación y asegurar la correcta identificación de los compuestos detectados. Para la elección de los iones productos, se tiene en cuenta tanto la sensibilidad (abundancia) como la selectividad (especificidad) del ion seleccionado.

3. Estudio de las condiciones óptimas de separación cromatográfica mediante la inyección de patrones en solvente: composición de la fase móvil (disolvente orgánico y modificadores), gradiente y volumen de inyección, que permitan obtener picos cromatográficos estrechos y con tiempos de retención adecuados.

4. Estudio del efecto matriz en los métodos LC-MS/MS desarrollados mediante la medida de patrones en solvente y de extractos de muestra fortificados: uso de patrones internos marcados isotópicamente para la corrección del efecto matriz.

5. Validación de los métodos optimizados evaluando la linealidad, exactitud y precisión mediante ensayos de recuperación en muestras fortificadas a varios niveles de concentración. Estimación del límite de cuantificación (LOQ) del método.

6. Aplicación de las metodologías analíticas desarrolladas para el análisis de aguas de efluente y superficial, y para piensos de distinta naturaleza.

7. Confirmación de la identidad de los compuestos detectados en las muestras siguiendo las guías y directrices internacionalmente aceptadas, evaluando la relación iónica (relación de intensidades para las transiciones seleccionadas) en muestras y patrones, así como el tiempo de retención.

8. Discusión de los resultados obtenidos y establecimiento de conclusiones relativas a la presencia de positivos en las muestras analizadas.

Metodología seguida en **métodos cualitativos**:

1. Experimentos en el laboratorio: (a) Ensayos de degradación en condiciones controladas. (b) Ensayos de metabolismo y excreción en orina.

2. Inyección de las muestras sometidas a ensayos de laboratorio en el sistema UHPLC-QTOF MS y adquisición del espectro de masas con medidas de masa exacta en modo MS^E, que implica la adquisición simultánea a baja y alta energía de colisión.

3. Procesamiento de los datos con el software específico de *Waters Corp.* (MetaboLynx XS™). Dicho software permite comparar cromatogramas del ion extraído (XICs) de una muestra "tratada" con una muestra control, de modo que los

picos diferenciales observados pueden atribuirse a posibles TPs/metabolitos generados.

4. Estudio de los espectros a baja y a alta energía de colisión (MS^E):

a. El espectro de baja energía proporciona información sobre la molécula (des)protonada, lo cual permite el cálculo de sus posibles composiciones elementales en base a su masa exacta.
b. El espectro de alta energía proporciona información sobre los iones fragmento en masa exacta, útil para obtener la estructura de los mismos.

5. Estudio de la fragmentación mediante análisis adicionales en modo MS/MS con un analizador QTOF MS, en casos de duda sobre la identidad del metabolito/TP, obteniendo los espectros de masas de los iones producto.

6. Identificación tentativa de los metabolitos/TPs generados en los ensayos de laboratorio sobre la base de toda la información suministrada en los análisis por QTOF MS.

7. Inyección del patrón de referencia, en caso de disponibilidad, y comparación de sus masas exactas, iones fragmento, perfil isotópico y tiempo de retención, con el fin de confirmar el metabolito/TP tentativamente identificado.

8. Investigación de la presencia de los metabolitos/TPs, formados en los ensayos de laboratorio, en muestras de agua de distintos tipos mediante análisis basados en LC-QTOF MS.

9. En la validación del método de *screening* aplicado a piensos, estimación del límite de detección del *screening* (SDL) y del límite de identificación (LOI) mediante análisis de distintos tipos de piensos fortificados con una mezcla de 116 fármacos a tres niveles de concentración.

El **plan de trabajo** seguido en esta Tesis Doctoral se detalla a continuación:

1. Selección de los compuestos a estudiar en base al consumo estimado en España (según información proporcionada por el Ministerio de Sanidad), a sus efectos potencialmente negativos en los organismos vivos del medio acuático y a la frecuencia de detección en aguas (según datos reportados en literatura científica).

2. Revisión bibliográfica sobre el estado actual de los métodos de análisis existentes para la determinación de fármacos y drogas de abuso mediante la técnica LC-MS/MS.

3. Desarrollo y optimización de un método analítico multi-residual rápido, y sensible, basado en la determinación mediante LC-MS/MS QqQ en aguas de diferentes tipos (residual urbana y superficial). Validación del método desarrollado y aplicación a aguas superficiales y residuales de efluente recogidas en diferentes puntos y EDARs de la Comunidad Valenciana.

4. Realización de ensayos de degradación en condiciones controladas en el laboratorio: hidrólisis, biodegradación, foto-degradación (ultravioleta o simulación de la luz solar) y cloración.

5. Realización de ensayos de metabolismo del omeprazol en orina humana, con el fin de investigar la identidad y la abundancia de los metabolitos excretados.

6. Desarrollo de una estrategia analítica basada en la adquisición en modo de espectro de masas completo y con medidas de masa exacta mediante QTOF MS para la identificación de los TPs/metabolitos generados en los experimentos desarrollados en los apartados 4 y 5.

7. Búsqueda retrospectiva de los metabolitos y TPs en muestras de aguas medioambientales y residuales mediante SPE y posterior determinación por LC-QTOF MS.

8. Desarrollo de métodos LC-MS/MS QqQ, utilizando la información proporcionada en los análisis con QTOF, para la determinación de metabolitos/TPs en aguas a nivel de trazas.

9. Búsqueda retrospectiva de metabolitos y TPs en aguas de efluente y aguas superficiales, previamente analizadas por UHPLC-QTOF MS en modo MS^E, aplicando la estrategia basada en la búsqueda de iones fragmento comunes.

10. Desarrollo y validación de un método de *screening* mediante UHPLC-QTOF MS, para la determinación cualitativa y semi-cuantitativa de 116 fármacos de uso animal y humano en muestras de pienso.

11. Aplicación de la metodología analítica descrita en el apartado anterior en muestras de pienso animal de distinta naturaleza.

12. 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 Doctoral Thesis is to explore the analytical capabilities of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), using triple quadrupole (QqQ), quadrupole time-of-flight (QTOF) and LTQ Orbitrap analyzers, for investigation of pharmaceuticals, drugs of abuse and their metabolites/transformation products in waters and animal feeds. With this research we pretend to improve the knowledge about environmental problems related with the presence of emerging contaminants in the aquatic environment as well as the issue of veterinary drugs in food safety.

On the one hand, the aim is to develop, validate and apply advanced analytical methodology based on UHPLC-MS/MS QqQ for pharmaceuticals and drugs of abuse frequently consumed in different types of waters.

On the other hand, the investigation on metabolites and transformation products in waters for some compounds scarcely detected in waters, despite their high consumption, is pursued making use of LC-QTOF MS and LC-MS/MS QqQ.

Finally, the study of LC-QTOF MS potential for screening and semi-quantitative evaluation of human and veterinary pharmaceuticals in animal feed samples is also pursued.

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

1. Development of advanced analytical methodology for multi-residue determination of pharmaceuticals and drugs of abuse frequently consumed, based on direct water sample injection into UHPLC-MS/MS with last generation triple quadrupole analyzer.

2. Application of the developed analytical methodology to wastewater and surface samples from different points of the Valencian Region. Comparison of the results with the on-line solid phase extraction (SPE) LC-MS/MS QqQ methodology applied in routine analysis by an ISO 17025 accredited laboratory specialized in environmental analysis.
3. Degradation studies, under laboratory controlled conditions, in order to simulate hydrolysis and sunlight photo-degradation processes occurring in the aquatic environment, and to elucidate the main transformation products generated.
4. Degradation study trying to simulate the processes typically occurring in a wastewater treatment plant: hydrolysis, biodegradation, chlorination and ultraviolet photo-degradation.
5. Development of analytical methodology based on LC-QTOF MS for identification and structural elucidation of transformation products generated after degradation experiments performed in the laboratory.
6. Application of the developed methodology to emerging compounds highly consumed, some of them scarcely detected in waters, such as omeprazole, venlafaxine, ibuprofen, gemfibrozil, ofloxacin, irbesartan as well as the main metabolite of cannabis, THC-COOH.
7. Investigation of omeprazole metabolism and excretion by analysis of urine samples from three volunteers by UHPLC-QTOF MS, in order to detect, identify and elucidate important metabolites not previously reported.
8. Retrospective analysis of water samples previously analyzed by UHPLC-QTOF MS, in order to investigate the presence of TPs and metabolites previously identified in laboratory experiments.

9. Development of analytical methodology based on LC-MS/MS (QqQ) for the quantitative determination of metabolites and TPs in water samples on the basis on the information provided by UHPLC-QTOF MS ((de)protonated molecule and fragment ions observed).

10. Investigation on the presence of metabolites/TPs in wastewaters and surface waters by LC-QTOF MS using the methodology based on *common fragment ions* searching.

11. Development of an analytical screening method based on UHPLC-QTOF MS for 116 human and veterinary pharmaceuticals in animal feeds and application to different types of feed, as for goat, bovine, rabbit, poultry and pork.

Methodology and working plan

The methodology followed in this Thesis to develop the analytical methods involved in this Thesis could be separated in two well differentiated parts:

Quantitative methods:

1. Literature search and selection of possible analytes based on their reported consumption, their dangerousness/toxicity and potential presence in the aquatic environment.

2. Optimization of MS and MS/MS conditions by injection of analytical reference standards.

   a. Acquisition of the MS spectra under *scan* mode to establish the ionization mode and the optimum cone voltage for each analyte.
   b. Acquisition under *product ion scan* mode, after isolating the precursor ion, to optimize the collision energy and obtain the characteristic product ions.
   c. Selection of 3 MS/MS transitions per compound to facilitate its determination and assure the correct identification of the detected compounds. The selection of the most appropriated product ions was made based on its sensitivity (abundance) and its selectivity (specify of the transition).

3. Optimization of the chromatography separation by injecting reference standard solutions: mobile phase composition (organic solvent and modifiers), gradient and injection volume, trying to obtain suitable chromatographic peaks and retention times.

4. Study of the matrix effect in the LC-MS/MS developed methods using standards in solvent as well as spiked sample extracts. Use of isotopically labelled internal standards to correct matrix effects.

5. Validation of the optimized methods evaluating the linearity, accuracy and precision from recovery experiments in samples spiked at different concentration levels. Estimation of the limit of quantification (LOQ) of the method.

6. Application of the developed analytical methodologies to the analysis of effluent wastewater and surface water, and/or to different types of animal feed.

7. Confirmation of the identity of the compounds detected in the samples following the internationally accepted Guidelines and Directives, evaluating the ion ratio (intensity of the signal for the selected transitions) and retention time in samples and standards.

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

Qualitative methods:

1. Laboratory experiments: (a) Degradation experiments under controlled conditions. (b) Metabolism and excretion assays in urine.

2. Injection of the degraded samples in the UHPLC-QTOF MS system. Acquisition of the accurate-mass spectrum in MS^E, with the simultaneous acquisition at low and high collision energy.

3. Data processing using the specific software from *Waters Corp.* (MetaboLynx XS™). This application manager compared eXtracted Ion Chromatograms (XICs) of the analyte sample and the control sample, and highlights differences in the presence of compounds, which could be attributed to TPs/metabolites generated.

4. Study of the low and high collision energy (MS^E) spectra:

   a. The low energy spectrum provides information of the (de)protonated molecule, allowing to obtain its possible elemental composition according to its exact mass.
   b. The high energy spectrum provides information on fragment ions in exact mass.

5. In cases of doubt about the metabolite or TP identify, study of fragmentation by performing additional MS/MS experiments in QTOF MS, obtaining accurate-mass product ions spectra.

6. Tentative identification of metabolites/TPs generated in laboratory based on the information provided by QTOF MS analysis.

7. If the commercial standard was available, injection and comparison of its exact mass, fragment ions, isotopic profile and retention time, in order to unequivocally identify the compound detected in samples.

8. Investigation on the TPs/metabolites generated under laboratory experiments in different types of waters by LC-QTOF MS.

9. Validation of the screening method applied to animal feeds, estimating the screening detection limit (SDL) and the limit of identification (LOI) based on the analysis of different types of feed fortified with 116 pharmaceuticals at three concentration levels.

The **working plan** to reach the objectives pursued in this Thesis was the following:

1. Selection of the compounds to be studied according to the consumption data in Spain (based on the information provided by the Spanish Ministry of Health), the potential negative effects on the aquatic environment and the reported frequency of detection in waters (based on scientific literature data).

2. Bibliographic revision on analysis methods reported for the determination of pharmaceuticals and drugs of abuse by LC-MS/MS.

3. Development and optimization of a fast and sensitive LC-MS/MS QqQ multi-residue method in different types of water matrices (wastewater and surface water). Validation of the optimized method and application to SW and EWW samples collected from different points and different wastewater treatment plants (WWTPs) of Valencian Region.

4. Degradation experiments under laboratory controlled conditions: hydrolysis, biodegradation, photo-degradation (ultraviolet and sunlight simulation) and chlorination.

5. Omeprazole metabolism assays by analysis of human urine samples, in order to investigate the identity and abundance of the excreted metabolites.

6. Development of an analytical strategy based on *accurate-mass full acquisition* by QTOF MS analyzer, in order to identify and elucidate the TPs/metabolites generated after the experiments carried out in points 4 and 5.

7. Retrospective search of metabolites and TPs in environmental and wastewater samples previously analyzed by SPE and posterior determination by LC-QTOF MS.

8. Development of LC-MS/MS QqQ methods for determination of metabolites/TPs in waters at trace levels, using the information provided by the QTOF MS analysis.

9. Searching for metabolites and TPs in effluent wastewater and surface, previously analyzed by QTOF in MS^E mode, applying the *common fragment ions* strategy for searching unknowns.

10. Development and validation of a UHPLC-QTOF MS screening method for qualitative and semi-quantitative determination of 116 human and veterinary pharmaceuticals in feeds.

11. Application of the analytical methodology described above to different types of animal feeds.

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

CAPÍTULO 1

INTRODUCCIÓN GENERAL

1.1 Investigación de contaminantes orgánicos emergentes en el medio ambiente

- 1.1.1 Compuestos emergentes
- 1.1.2 Fármacos
- 1.1.3 Drogas de abuso
- 1.1.4 Metabolitos
- 1.1.5 Productos de transformación

1.2 Problemática de los metabolitos y productos de transformación (TPs)

1.3 Tratamiento de muestra

1.4 Degradación de contaminantes orgánicos

1.5 Acoplamiento cromatografía líquida-espectrometría de masas (LC-MS)

- 1.5.1 Problemática analítica general
- 1.5.2 Cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS)
- 1.5.3 Cromatografía líquida acoplada a espectrometría de masas de alta resolución (LC-HRMS)
- 1.5.4 Efecto matriz en métodos LC-MS

1.1 Investigación de contaminantes orgánicos emergentes en el medio ambiente

El agua es un recurso natural escaso, indispensable para la vida humana y el sostenimiento del medio ambiente, que, como consecuencia del rápido desarrollo humano y económico y del uso inadecuado que se ha hecho de él como medio de eliminación, ha sufrido un alarmante deterioro. Durante décadas, toneladas de sustancias biológicamente activas, sintetizadas para su uso en la agricultura, la industria, la medicina, etc., han sido vertidas al medio ambiente sin reparar en las posibles consecuencias. Al problema de la contaminación, que comenzó a hacerse notable ya a principios del siglo XIX, cabe añadir el de la escasez, aspecto que está adquiriendo proporciones alarmantes a causa del cambio climático y la creciente desertización que está sufriendo el planeta (Catalán, 1969).

Las medidas legislativas que se han ido adoptando progresivamente para evitar la contaminación química del agua y los riesgos que se derivan de ello, han contribuido a paliar parcialmente esta situación. Sin embargo, la creciente demanda de agua y el continuo descubrimiento de nuevos contaminantes potencialmente

peligrosos, dejan clara la necesidad de seguir investigando en todas aquellas áreas que puedan contribuir a proteger la salud humana y el medio ambiente, además de conseguir un uso sostenible del agua y atenuar los efectos de las sequías y del cambio climático.

Actualmente, la Directiva 2013/39/UE regula las sustancias prioritarias en el ámbito de la política de aguas. Esta directiva modifica la DMA (Directiva Marco del Agua 2000/60/EC) y la EQSD (*Environmental Quality Standards Directive*), y amplía la lista hasta 45 sustancias prioritarias, de las cuales 21 son identificadas como peligrosas.

Con esta nueva Directiva Europea, aprobada en el mes de agosto de 2013, ya no es suficiente tratar las aguas con las tecnologías convencionales para su depuración, sino que es necesario dar un paso más y trabajar en el desarrollo de tecnologías emergentes avanzadas (Lapworth, 2012).

1.1.1 Compuestos emergentes

Hace poco más de diez años, empezó a postularse el concepto de contaminante emergente, como consecuencia de la aparición, en los ríos, de numerosas sustancias que entonces aún no se consideraban contaminantes. Bajo esta etiqueta no sólo se incluyeron fármacos, sino todas las nuevas moléculas que potencialmente podían tener efectos en el medio ambiente y que no estaban reguladas (Stuart, 2012). Ejemplos de ellos son fármacos, drogas de abuso, productos de higiene personal (detergentes, desodorantes), filtros ultravioleta utilizados en cremas solares, o fragancias sintéticas utilizadas en productos de limpieza.

Muchos de estos compuestos se usan diariamente. Algunos de ellos, son directamente consumidos por el ser humano. Tras ser ingeridos, son excretados llegando, a través de los saneamientos, a las estaciones depuradoras de agua

residual (EDARs). Allí dichos compuestos pueden no ser eliminarlos adecuadamente, alcanzando finalmente el medio ambiente (Li, 2014; Meffe, 2014).

El hecho de que el agua es capaz de transmitir enfermedades es muy antiguo. Por ejemplo, Hiprócrates ya recomendaba la ingestión del agua hervida con objeto de evitar enfermedades. La ley persa recomendaba que el agua de bebida se conservase en vasijas de cobre brillante. Heródoto narra cómo el agua destinada al rey de Persia, recogida del rio Choaspes, era hervida y almacenada en botes. Los egipcios, en la primera centuria antes de Cristo, trataban el agua de bebida por filtración. Y Aristóteles cita el uso de un filtro de porcelana sin vidriar para filtrar el agua de bebida.

De aquí el gran esfuerzo científico que se ha hecho y se está realizando en la actualidad, tanto para el reconocimiento de contaminantes causantes de enfermedades, como para desarrollar técnicas que permitan suministrar agua para el consumo humano, garantizando que está libre de toda contaminación (Stuart, 2012; Samra, 2013).

Una solución para evitar que gran cantidad de compuestos emergentes lleguen al medio ambiente podría ser mejorar las estaciones depuradoras, incluyendo tratamientos terciarios de última generación. Actualmente, cada depuradora aplica sus propios tratamientos, según crea conveniente. Es importante resaltar que no hay un desinfectante universal que sea capaz de eliminar todos los contaminantes presentes en un agua. Por ello, la elección del agente desinfectante depende normalmente de la naturaleza de los compuestos a eliminar, así como de su concentración y su estado (Stuart, 2012).

En esta Tesis se han estudiado diferentes tipos de compuestos emergentes, entre los que destacan fármacos, tanto de uso humano como de uso veterinario, drogas de abuso, así como sus metabolitos y sus productos de transformación. Se han tratado no solamente aspectos analíticos, relacionados con su determinación a niveles de sub-ppb, sino también la degradación de compuestos seleccionados en experiencias de laboratorio y la presencia de los productos de transformación más relevantes en el medio ambiente acuático.

1.1.2 Fármacos

Son sustancias químicas de origen natural o sintético, capaces de modificar alguna función de los seres vivos. Se utilizan para la prevención, diagnóstico, tratamiento y cura de enfermedades.

Constituyen un grupo de compuestos muy numeroso (alrededor de 3000), pudiéndose clasificar según su origen, estructura o mecanismo de acción (Richardson, 2008). Entre ellos, los grupos de fármacos que en la actualidad se consideran más peligrosos para el medio ambiente y, para los que se requiere intensificar la investigación son:

- Antibióticos: Pueden desarrollar cepas bacterianas resistentes, haciendo que estos compuestos resulten ineficaces para el fin que fueron diseñados.

- Medidores de contraste en rayos X: Son muy persistentes, no siendo eliminados en las plantas depuradoras y por consiguiente, alcanzando fácilmente las aguas.

- Citostáticos: Tienen una gran potencia farmacológica, exhibiendo con frecuencia propiedades carcinogénicas, mutagénicas o embriogénicas.

- Estrógenos: Pueden ser los responsables de la aparición de fenómenos de feminización, hermafroditismo y disminución de la fertilidad.

El término "fármaco" engloba tanto aquellos compuestos utilizados principalmente en tratamientos a humanos, como los destinados a uso veterinario.

En la presente Tesis se han estudiado más de 40 **fármacos de uso humano** pertenecientes a diferentes familias: acetaminofén, ibuprofeno, ácido salicílico, naproxeno, diclofenaco (utilizados como ***analgésicos y antiinflamatorios***), 4-acetil-aminoantipirina (4-AAA), 4-formil-aminoantipirina (4-FAA), 4-aminoantipirina (4-AA) (metabolitos del analgésico metamizol), atorvastatina, pravastatina, bezafibrato, gemfibrozil (***reguladores lipídicos***), olanzapina (***antipsicótico***), venlafaxina, carbamazepina (***antidepresivos***), alprazolam, lorazepam (***ansiolíticos***), omeprazol, pantoprazol (***antiulcerosos***), enalapril, valsartán e irbesartán (***antihipertensivos***) (**Figura 1.1**).

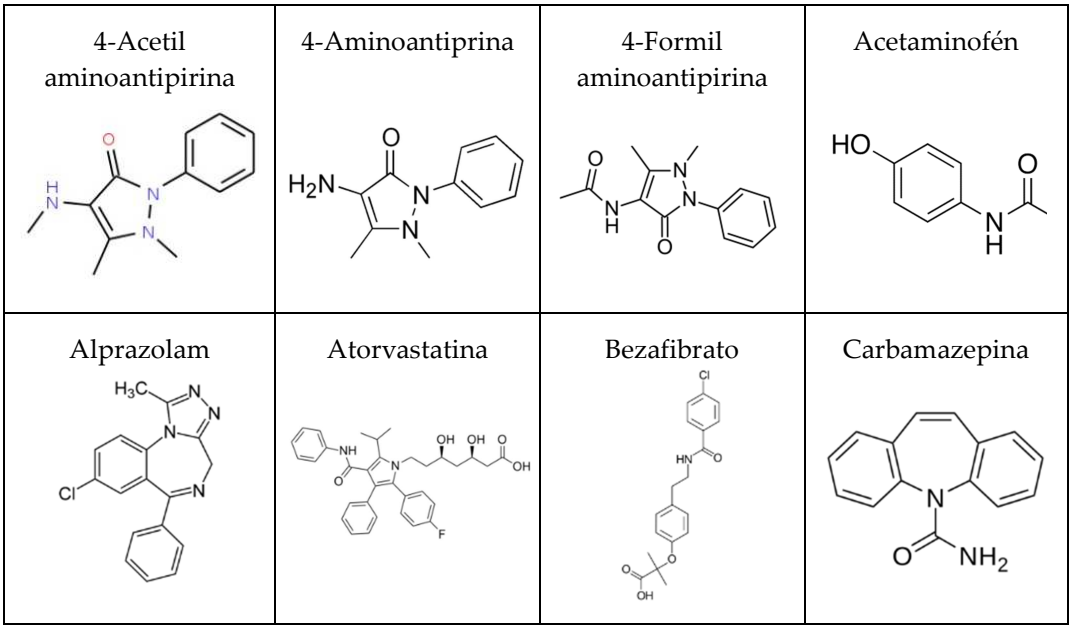


Figura 1.1 Estructuras químicas de fármacos de uso humano estudiados en esta Tesis.

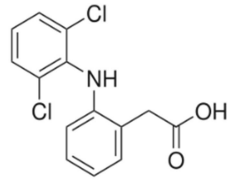
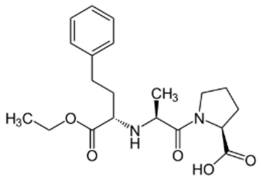
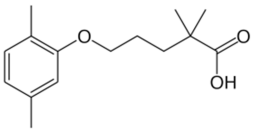
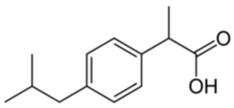
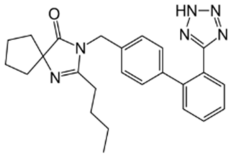
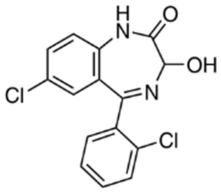
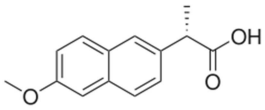
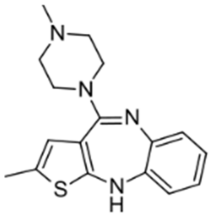
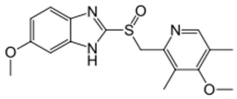
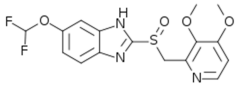
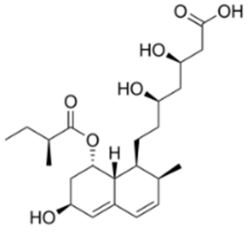
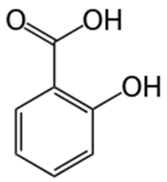
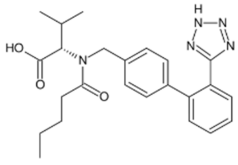
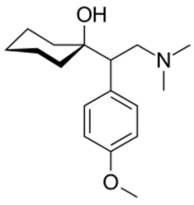
Diclofenaco 	Enalapril 	Gemfibrozil 	Ibuprofeno 
Irbesartán 	Lorazepam 	Naproxeno 	Olanzapina 
Omeprazol 	Pantoprazol 	Pravastatina 	Ácido Salicílico 
Valsartán 	Venlafaxina 		

Figura 1.1 (Cont.) Estructuras químicas de fármacos de uso humano estudiados en esta Tesis.

Como ya se ha apuntado, los ***antibióticos*** es uno de los grupos de fármacos más importantes. Se utilizan para tratar infecciones provocadas por gérmenes, ya que impiden el crecimiento de cierta clase de organismos sensibles, principalmente

bacterias. Algunos ejemplos de antibióticos estudiados en esta Tesis son: ofloxacino, ácido nalidíxico, ácido oxolínico, flumequina (quinolonas), eritromicina, claritromicina, roxitromicina (macrólidos), sulfametoxazol, sulfadiazina, sulfadoxina (sulfonamidas), lincomicina, furaltadona y trimetoprima (lincosamidas) (**Figura 1.2**).

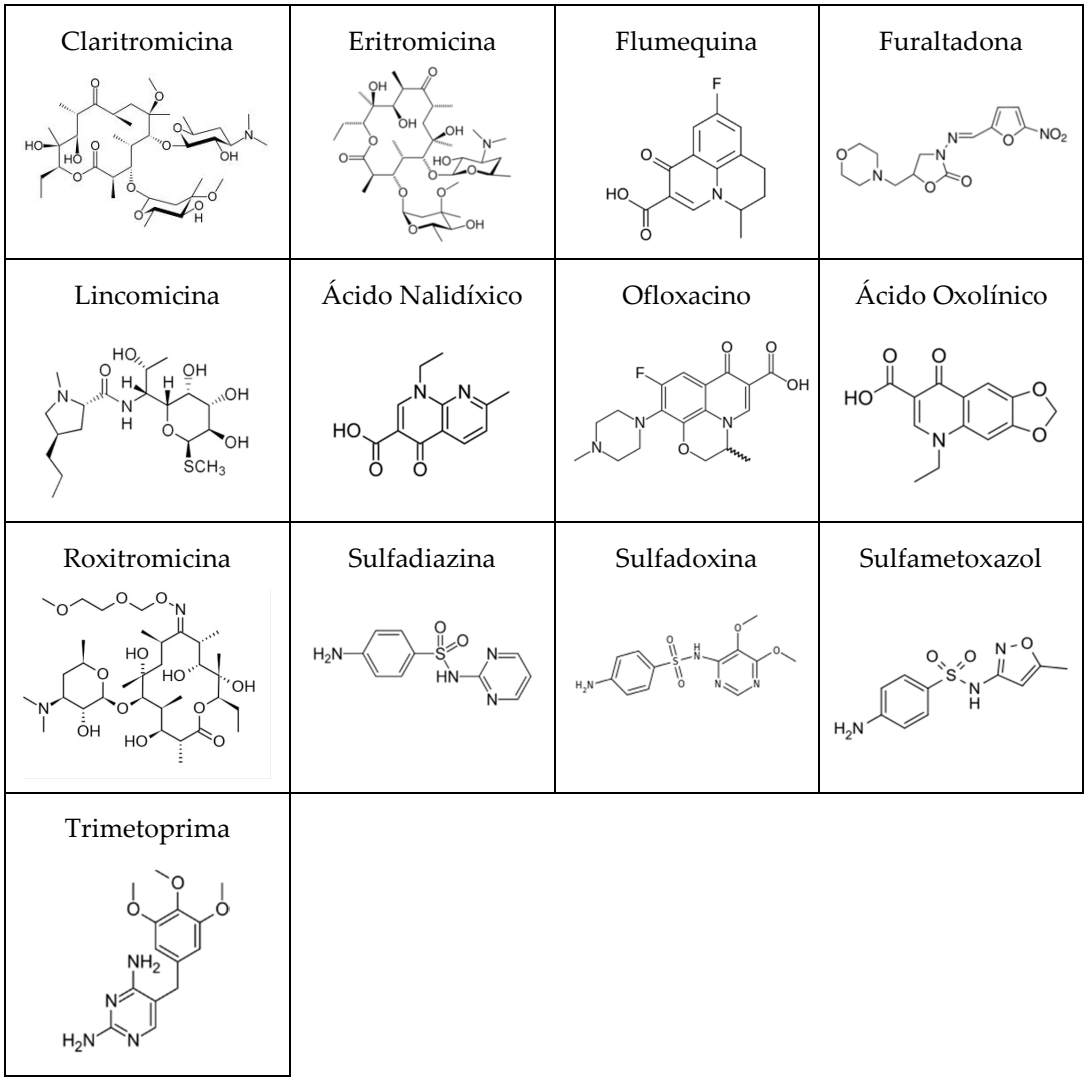


Figura 1.2 Estructuras químicas de antibióticos de uso humano estudiados en esta Tesis.

Es importante tener en cuenta la problemática de los fármacos en estudios medioambientales, puesto que algunos como el ácido salicílico, el diclofenaco o la carbamazepina se han detectado en muestras de efluente a concentraciones consideradas perjudiciales para la vida de los peces, los organismos bentónicos o para el zooplancton (Richardson, 2014). Por este motivo, una parte importante de la Tesis se ha centrado en investigar la presencia de fármacos y algunos de sus metabolitos y TP en el medio ambiente acuático.

Por otro lado, también se han tenido presente en esta Tesis los **fármacos destinados al tratamiento animal**. Según el artículo 1 de la Directiva 2004/28/CE, "medicamento veterinario" es toda sustancia o combinación de sustancias que posee propiedades curativas o preventivas respecto a las enfermedades animales; o que puede administrase al animal con el fin de restablecer, corregir o modificar sus funciones fisiológicas, ejerciendo una acción farmacológica, inmunológica o metabólica.

Los principales grupos de medicamentos usados con fines terapéuticos en animales y que pueden originar residuos son, los antimicrobianos, las quinolonas, los macrólidos o las β-lactamas.

La Directiva del Consejo 96/23/CE, de 29 de abril, relativa a medidas de control aplicables respecto de determinadas sustancias y sus residuos en animales vivos y sus productos, establece y diferencia dos grupos de sustancias objeto de control, denominadas A y B (Anexo I):

- Grupo A: Sustancias con efecto anabolizante y sustancias no autorizadas.
- Grupo B: Sustancias antibacterianas, incluidas las sulfamidas, quinolonas, otros medicamentos veterinarios y contaminantes medioambientales.

El Anexo II de la Directiva del Consejo 96/23/CE, establece el grupo de residuos o sustancias objeto de control según el tipo de animal, así como el muestreo de piensos y agua de consumo, y de productos animales. De acuerdo con la Directiva, cada Estado miembro de la Unión Europea (*European Union*, EU) debe aprobar un plan de control oficial de residuos.

Las sustancias expresamente prohibidas en la EU para uso en animales productores de alimentos pueden clasificarse en diferentes categorías (Anexo II):

1. Hormonas anabolizantes y β-agonistas que se usen con fines de promoción del crecimiento. Las sustancias clasificadas en el Anexo IV del Reglamento (CEE) No 2377/90 (nitrofuranos (incluida furazolidona), ronidazol, dapsona, cloranfenicol, dimetridazol, colchicina, clorpromacina, cloroformo, metronidazol, *aristolochia spp*. y sus preparaciones) también están prohibidas en animales productores de alimentos en la EU, debido a que sus residuos en alimentos de origen animal constituyen un peligro para la salud del consumidor a cualquier concentración (Artículo 5, Reglamento (CEE) No 2377/90); es decir, se aplica el principio de tolerancia cero para estas sustancias.
2. Antibióticos aditivos promotores del crecimiento. Incluyen varios aditivos promotores del crecimiento, específicamente prohibidos para uso en animales productores de alimentos en la EU debido a que existen evidencias de la transferencia de resistencia de antibióticos a humanos. La EU ha prohibido a partir del 1 de enero de 2006 el uso de los siguientes antibióticos: avilamicina, flavofosfolipol y los antibióticos poliésteres ionóforos salinomicina y monensina, con algunas excepciones de uso como coccidiostáticos utilizados en producción aviar (ver Reglamento (CE) No 1831/2003 del Parlamento Europeo y

del Consejo, de 22 de septiembre de 2003 sobre los aditivos en la alimentación animal).

3. Sustancias no incluidas en los Anexos I, II y III del Reglamento del Consejo (CEE) No 2377/90, que no tienen establecido el nivel máximo de residuo (*Maximum Residue Level*, MRL). Estas incluyen: a) medicamentos veterinarios que contienen principios activos que no están autorizados para uso en otras especies de animales productores de alimentos (por ejemplo, tianfenicol, con un MRL para bovino, pero no para ovino), b) medicamentos veterinarios conteniendo principios activos que no tienen MRL y no están, por lo tanto, autorizados en especies de animales productores de alimentos (por ejemplo, fenilbutazona) y c) sustancias que nunca han sido autorizadas como medicamentos veterinarios, no tienen MRL, pero han sido usadas como medicamentos (por ejemplo, verde malaquita en acuicultura).

1.1.3 Drogas de abuso

Las drogas de abuso son sustancias químicas, psicoactivas o psicotrópicas, capaces de alterar el estado de conciencia, el humor, los sentimientos, la conducta, las motivaciones y los procesos del pensamiento del individuo que las consume, traduciéndose clínicamente en estimulación o depresión del sistema nervioso central.

Muchas de las drogas de origen natural ya eran utilizadas en Asia, América y África desde hace cientos de años, donde su consumo tenía fines místicos. Con el intercambio cultural que hubo por las conquistas europeas en otros territorios, el uso de sustancias tóxicas se extendió, cambiando sus fines. Después de la segunda guerra mundial, y especialmente durante la década de los sesenta, el uso de drogas

de origen natural se popularizó en todo el mundo. Poco después se sumó de forma abierta la producción, tráfico y consumo de drogas artificiales.

El hombre ha buscado en ellas la nutrición física, la cura para sus enfermedades, alimentar sueños o alcanzar el clímax, influenciar el humor o, buscar la paz o la excitación; en definitiva, abstraerse del mundo que le rodea y le perturba en cierto momento de su existencia.

El elevado y popular consumo de estas sustancias produjo importantes consecuencias sociales y de salud. Las listas de muertes o enfermedades graves por su uso se hicieron interminables y las redes del narcotráfico cada vez más poderosas. Producir y distribuir sustancias ilegales, desde entonces hasta hoy, ha resultado ser un importante negocio, que continúa a lo largo del tiempo a pesar de los graves daños que ocasionan.

Las drogas de abuso, por su uso ilegal, su fácil adicción y su conocida peligrosidad para la salud, son posiblemente el grupo de compuestos emergentes que está originando mayor alarma social. Por todo ello, el estudio de estos compuestos resulta de especial interés no sólo en el campo toxicológico sino también en el campo medioambiental. Además, el análisis de aguas residuales constituye una herramienta poderosa para estimar el consumo de drogas de una población, constituyendo una disciplina reciente, conocida como epidemiología de las aguas residuales (*sewage epidemiology*) (Thomas, 2012).

En esta Tesis se han estudiado seis **drogas de abuso** (incluyendo algunos metabolitos mayoritarios) de diferentes familias y de conocido elevado consumo (UNODC, 2013; EMCDDA, 2014): el principal metabolito del cannabis (11-nor-9-carboxi-delta-9-tetrahidrocannabinol, THC-COOH), cocaína y sus metabolitos

benzoilecgonina y cocaetileno, anfetamina y MDMA (3,4-metilendioximetanfetamina) (**Figura 1.3**).

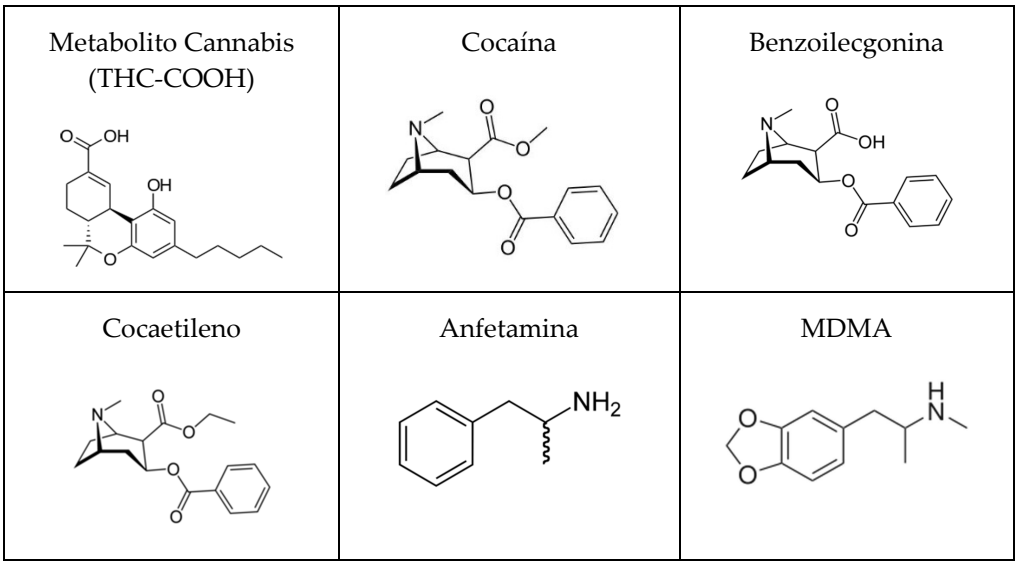


Figura 1.3 Estructuras químicas de las 6 drogas de abuso/metabolitos estudiados en esta Tesis.

1.1.4 Metabolitos

Un "metabolito" es cualquier sustancia producida durante la descomposición (metabolismo) por parte del cuerpo. La palabra metabolismo viene de la voz griega "*metabolé*" que quiere decir cambio, transformación. El metabolismo se podría definir como el conjunto de procesos físicos y químicos que tienen lugar en los seres vivos, que convierten o usan energía para transformar a las sustancias. Ejemplos de ellos son: la respiración, la circulación sanguínea, la regulación de la temperatura corporal, la contracción muscular, la digestión de alimentos y nutrientes, la eliminación de los desechos a través de la orina y de las heces, y el funcionamiento del cerebro y los nervios. Para que se sucedan todos estos cambios se deben cumplir tres fases generales:

1. La absorción de los compuestos: esta etapa consiste en la penetración de especies químicas a través de la membrana plasmática.

2. La fase de transformación: ésta abarca todos los procesos por los que el protoplasma transforma las especies químicas y la energía absorbida. Comprende la secreción, digestión, asimilación y desasimilación. Durante estas transformaciones, en el hígado, se suelen dar reacciones en dos fases:

Fase I: Suelen ser oxidaciones, reducciones o hidrólisis que introducen en la estructura un grupo reactivo que lo convierte en químicamente más activo (funcionalización).

Fase II: Suelen ser reacciones de conjugación, que por regla general inactivan al fármaco. Suelen actuar sobre el grupo reactivo introducido en la *Fase I* (con ácido glucurónico, sulfatos, acetatos, metilos, glutatión o aminoácidos).

3. La excreción: es la última etapa, en la que se lleva a cabo la eliminación de las especies químicas que no se han incorporado al protoplasma o se han dispersado en energía (calor, luz).

La generación/producción de metabolitos es una etapa muy difícil de controlar. Cada organismo genera una proporción diferente de metabolitos, pudiéndose incluso generar diferentes metabolitos de un mismo fármaco según el organismo.

Tras estas etapas, son muchas veces los metabolitos, y no el compuesto inalterado, quienes son excretados y llegan a las aguas residuales, cuando se trata de fármacos o drogas de abuso. Cuando estos metabolitos no son correctamente eliminados en las estaciones depuradoras, alcanzarán el medio ambiente. La toxicidad/peligrosidad de la mayoría de los metabolitos es desconocida, pudiendo ser perjudiciales tanto para la vida animal como humana. Ello hace, que el estudio

del comportamiento de los metabolitos sea uno de los campos que está experimentando mayor auge en los últimos años (Escher, 2011; Gracia-Lor, 2014).

1.1.5 Productos de transformación

Los "productos de transformación" (*Transformation Products*, TPs) son aquellos compuestos que se generan tras un cambio químico o físico del compuesto original, debido a las condiciones del medio en el que se hallen. Se diferencian de los metabolitos en que las transformaciones no tienen lugar en el cuerpo humano o animal.

En los últimos años, ha aumentado el interés de la comunidad científica por los productos resultantes tras los procesos de degradación/transformación, ya sean naturales, que pueden darse en el medioambiente, o forzados, aplicados en las plantas de depuración de aguas (Fatta-Kassinos, 2011b; Escher, 2011). Esta preocupación se acrecienta al observar la presencia de un significativo número de productos de transformación en las aguas.

Los TPs se generan comúnmente en las estaciones depuradoras, una vez se han aplicado los tratamientos generales:

1) Tratamiento primario: tiene como misión la separación por medios físicos de las partículas en suspensión y el agua.

2) Tratamiento secundario: el más comúnmente empleado para aguas residuales urbanas (*wastewater*, WW). Consiste en un proceso biológico aerobio donde se emplean fangos activos o lechos bacterianos, seguido por una decantación.

3) Tratamiento terciario: este tratamiento es el más completo y efectivo para las aguas residuales, pero no ha sido ampliamente adoptado por ser muy costoso. Se basa en procesos físico-químicos que disminuyen considerablemente los niveles de contaminantes en las aguas.

El funcionamiento de las plantadas depuradoras se explicará con más detalle en los Capítulos 3-4.

Por otro lado, también se pueden generar TPs en el medio ambiente, debido al efecto de la radiación solar, o mediante los microorganismos presentes en los lagos, ríos, suelos, aire,…

En esta Tesis, se ha estudiado la formación de TPs para compuestos seleccionados, sometidos a diferentes procesos: TPs de hidrólisis, TPs de foto-degradación (por ultravioleta (UV) o por simulación de la luz solar), TPs de biodegradación y TPs de cloración.

1.2 Problemática de los metabolitos y TPs

En los últimos tiempos ha aumentado la preocupación en torno a la presencia de fármacos y de drogas de abuso en las aguas residuales urbanas, lo que, combinado con los riesgos potenciales derivados de su posible transferencia al medio ambiente, pone de manifiesto la urgente necesidad de conocer su destino en el proceso de tratamiento de las aguas residuales.

Gracias a la mejora de las técnicas y estrategias analíticas, se ha detectado un amplio abanico de compuestos emergentes, muchas veces desconocidos en las aguas residuales (Ibáñez, 2005; Hernández, 2011). Aunque es notable la incertidumbre sobre los efectos potenciales de dichos compuestos en los ecosistemas acuáticos, el principio de precaución conducirá, en un futuro, a la probable aprobación de legislaciones más estrictas en cuanto al tratamiento de aguas residuales.

Las reacciones de metabolización y degradación que darán lugar a los metabolitos y a los productos de transformación respectivamente, suelen ser: oxidaciones, hidroxilaciones, hidrólisis, dealquilaciones o combinaciones de ellas. Dado que se suelen formar TPs/metabolitos más polares que los productos de partida, su movilidad y posibilidad de contaminar las aguas son generalmente mayores que para los compuestos inalterados.

Dependiendo del proceso al que sea sometido el compuesto de partida se formará un determinado TP/metabolito. Todos ellos deberían ser caracterizados físico-química y toxicológicamente. No obstante, debido a la gran cantidad de compuestos que se pueden generar y a la laboriosa y complicada tarea de su completa identificación, normalmente las investigaciones ambientales se centran en aquellos TPs/metabolitos más abundantes y sobretodo más persistentes. Sin embargo, muchas veces estos compuestos son aún desconocidos, y es labor de los químicos

analíticos el llevar a cabo su detección e identificación en el medio ambiente para poder, en una etapa posterior, evaluar su posible impacto negativo sobre el ecosistema acuático y sobre la salud pública.

Hasta la fecha se desconoce todavía la trascendencia de muchos de los productos de transformación o metabolitos en el medio ambiente, pero el enfoque de esta Tesis representa un paso útil para tratar de identificar compuestos aún desconocidos, que pueden ser causantes de determinados efectos observados en los ecosistemas acuáticos y terrestres.

1.3 Tratamiento de muestra

La mayoría de métodos multi-residuo aplicados para fármacos o drogas de abuso en muestras complejas como pueden ser aguas, suelos, alimentos o pienso, suele incluir una etapa de extracción de las muestras. Dicha etapa resulta especialmente útil cuando los analitos se encuentran a muy bajos niveles de concentración, consiguiendo, de ese modo, no sólo la pre-concentración de los analitos, para alcanzar la sensibilidad requerida, sino también la eliminación de algunos de los posibles interferentes.

El tratamiento de muestra que se selecciona está directamente relacionado con las características de la matriz, el tipo de analito que se desea extraer y la técnica de determinación que se pretende aplicar.

En aquellos métodos donde se incluye un gran número de analitos, resulta casi imposible obtener una metodología de extracción óptima para todos ellos. Por ello, en la mayoría de situaciones es necesario llegar a una situación de compromiso, manteniendo en el método algunos de ellos, aun sabiendo que los resultados obtenidos serían mejorables con otro procedimiento dirigido hacia estos analitos más problemáticos.

Los tratamientos de muestra utilizados en la presente Tesis han sido:

1. Extracción con solventes. Es la aproximación más usual para el tratamiento de muestras sólidas. Se han utilizado disolventes orgánicos como el acetonitrilo, acetato de etilo, metanol o acetona. En este proceso de extracción se hace uso de un sistema de homogenización entre disolvente y muestra para aumentar la efectividad. Suelen realizarse con

ultraturrax o agitación en brazos rotores, aunque en algunos casos se han llegado a usar otros sistemas como el ultrasonidos.

2. Extracción en fase solida (*Solid Phase Extraction*, SPE). Es el tratamiento de muestra más utilizado en matrices acuosas para alcanzar la sensibilidad deseada. La SPE puede realizarse de dos modos: "*off-line*" y "*on-line*". El primero de ellos es el que se ha utilizado en esta Tesis. Consiste en la utilización de cartuchos, habitualmente desechables, donde se atrapan los analitos gracias a una fase adsorbente apropiada. Los compuestos retenidos son posteriormente eluidos con un disolvente adecuado. Habitualmente, se introduce una etapa final de evaporación y reconstitución previa a la inyección en el sistema, con el fin de hacer el extracto compatible con el sistema cromatográfico utilizado.

   Esta técnica se considera versátil y fiable, y permite ajustar fácilmente el volumen de agua extraída y las condiciones de elución con el fin de obtener la sensibilidad deseada y maximizar las recuperaciones de los compuestos (Bagnati, 2011).

3. Inyección directa. En este caso, después de una simple centrifugación, filtración o ajuste del pH, la muestra se inyecta directamente en el sistema cromatográfico. Las principales ventajas de este tratamiento respecto a otras aproximaciones son el menor tiempo de análisis así como la escasa manipulación de muestra, lo que disminuye la posibilidad de que se produzcan errores por pérdida de analito o por contaminación de la muestra.

Estas tres aproximaciones se han utilizado en el desarrollo experimental de esta Tesis en función de la matriz estudiada (agua o pienso), de los niveles de concentración requeridos y de la sensibilidad del equipo analítico disponible.

1.4 Degradación de contaminantes orgánicos

Los tratamientos específicos que se llevan a cabo en cada estación depuradora, así como el constante cambio de los procesos de depuración para aumentar su eficacia, hacen complicado el estudio de los productos que se generarán. Aunque existen estudios que han aportado gran conocimiento en el campo de los productos de transformación, aún queda mucho por investigar (Escher 2011; Fatta-Kassinos, 2011b).

En esta Tesis se diferencian dos tipos de transformación/degradación. Por un lado, las transformaciones que pueden tener lugar en las estaciones depuradoras, con tratamientos controlados (biodegradación, foto-degradación ultravioleta y cloración); y por otro lado, las que se dan, de forma natural, en el propio medio ambiente (hidrólisis, biodegradación y foto-degradación por simulación de la luz solar).

Reacciones de Hidrólisis: tienen lugar cuando los compuestos orgánicos interactúan con el agua. Se caracterizan por la división de una molécula de agua en los grupos hidrógeno e hidróxido, uniéndose estos átomos a la molécula orgánica y pasando así a formar parte de otra especie química. Esta reacción es importante por el gran número de contextos en los que el agua actúa como disolvente.

Dicho intercambio puede tener lugar tanto en el medio ambiente como en estaciones depuradoras.

Reacciones de Foto-degradación: tienen lugar por rupturas de enlaces químicos a causa de energía radiante. Se produce por la interacción de uno o más fotones con una molécula objetivo. Es una reacción fotoquímica en la que una molécula adsorbe un quantum de luz y se disocia dando lugar a una o varias sustancias más simples.

Los fragmentos producidos son, a veces, átomos o radicales libres, por lo que esta reacción se da generalmente en un mecanismo en cadena. La energía de un *quantum* de luz debe ser igual o mayor que la energía de disociación de la molécula, y por tanto es frecuente la formación de un átomo en estado excitado (es poco probable que el *quantum* de luz proporcione exactamente la energía de disociación de la molécula en su estado fundamental).

Por ejemplo, la foto-degradación de agua, ocurre por descomposición de la molécula de agua en sus elementos constituyentes (H y O) tras la acción de la luz. Se representa de la siguiente manera:

$$2\ H_2O + 2\text{fotones} \longrightarrow 4\ H^+ + O_2 + 4\ e^-$$

Los procesos de foto-degradación estudiados en esta Tesis son:

a) Aquellos que simulan radiaciones solares, que comprenden longitudes de onda en el rango de 300-800 nm. Este tipo de foto-degradación tendría lugar mayoritariamente en el medio ambiente.
b) Radiaciones ultravioleta, son radiaciones electromagnéticas cuya longitud de onda está comprendida aproximadamente entre los 400 nm ($4x10^{-7}$ m) y los 15 nm ($1.5x10^{-8}$ m). Son procesos forzados, que se darían en las estaciones depuradoras como un tratamiento de desinfección.

Reacciones de Biodegradación: son procesos naturales por los que determinadas sustancias pueden ser descompuestas con cierta rapidez en sus ingredientes básicos, debido a la acción de bacterias, levaduras y otros hongos microscópicos existentes en el suelo y en el agua. La descomposición puede llevarse a cabo en presencia de oxigeno (aeróbica) o en su ausencia (anaeróbica). La primera es más completa y libera energía, dióxido de carbono y agua, siendo la de mayor rendimiento

energético. Los procesos anaeróbicos son oxidaciones incompletas y liberan menor energía.

Los TPs de biodegradación pueden tener lugar tanto en el medio ambiente (por acción de microorganismos naturales en las aguas), como en las estaciones depuradoras (por acción de lodos activados).

Reacciones de Cloración: la cloración es uno de los procedimientos de desinfección de aguas utilizados en las plantas depuradoras. Como reactivo se puede emplear gas cloro, pero normalmente se utiliza hipoclorito de sodio (lejía) por su menor toxicidad, mayor facilidad de almacenamiento y dosificación. Éste reacciona eliminando las bacterias y los virus, causando alteraciones físicas, químicas y bioquímicas en la pared de toda célula. Además, el hipoclorito sódico (NaOCl), es un compuesto fuertemente oxidante, que contiene el cloro en estado de oxidación (+1), por ello, su uso frecuente como desinfectante y blanqueador.

1.5 Acoplamiento cromatografía líquida-espectrometría de masas

1.5.1 Problemática analítica general

Con el fin de garantizar la calidad de las determinaciones analíticas es necesario obtener datos de concentración fiables, e incluso todavía más importante, evitar reportar tanto falsos positivos como falsos negativos. Para ello, parámetros fundamentales son la exactitud, la precisión y la especificidad de la determinación. La utilización de técnicas analíticas poderosas, de última generación, facilita la correcta cuantificación, además de minimizar las posibles interferencias, pero aun así son necesarios importantes esfuerzos para minimizar las fuentes de error en los análisis

Las primeras separaciones analíticas por medio de métodos cromatográficos fueron llevadas a cabo por Twetts en 1906. Este botánico ruso consiguió separar algunos pigmentos coloreados de hojas de plantas utilizando una columna de alúmina. El desarrollo de estas técnicas se produjo, no obstante, a partir del año 1931, cuando Kuhn y Lederer comenzaron a utilizarlas de forma sistemática. La cromatografía de líquidos (*Liquid Chromatography*, LC) sufrió un relativo estancamiento a partir de 1952, siendo la de gases (*Gas Chromatography*, GC) la que acaparó los esfuerzos teóricos encaminados al conocimiento profundo de la cromatografía. No obstante, las limitaciones de la GC en cuanto al tipo de analitos (volátiles, de baja polaridad y/o estables térmicamente) hicieron que a finales de los sesenta se volviera a considerar la LC, que presenta un rango más amplio de aplicación sin limitación por la volatilidad o la estabilidad térmica de los analitos.

La mayoría de contaminantes emergentes son compuestos de elevada polaridad. Además, la mayoría de los contaminantes que se encuentran en el medio ambiente sufren transformaciones por vía química, fotoquímica o bioquímica, originando

compuestos relativamente estables y en general más polares que el compuesto original. Por esta razón, la cromatografía líquida resulta una herramienta ideal para la determinación de la mayoría de moléculas polares, incluso iónicas, como son los TPs o metabolitos.

La cromatografía líquida es una técnica separativa basada en la afinidad que los componentes tienen entre dos fases inmiscibles: una fase móvil, líquida, que fluye sobre otra estacionaria, sólida, empaquetada en una columna. La fase móvil se compone generalmente de una mezcla de disolventes con distinta fuerza eulotrópica. La separación es una consecuencia de la diferencia de coeficientes de distribución entre los componentes de una muestra, que produce sucesivas etapas de adsorción y desorción de los mismos a través de la fase estacionaria.

Aunque existen diversos tipos de LC, esta Tesis se centra en la LC en fase reversa, ya que ha sido el tipo de fase estacionaria utilizado en todos los trabajos realizados. Las columnas de fase reversa se adaptan bien al análisis de compuestos polares. El tipo de material apolar con que se rellenan dichas columnas suele ser sílice químicamente modificada (cadenas de C8 y C18). Como fase móvil, se utilizan disolventes con cierta polaridad, generalmente combinaciones de agua con mezclas de disolventes orgánicos como metanol o acetonitrilo. El porcentaje y tipo de modificador orgánico en la fase móvil es el factor más determinante en la retención de los analitos polares. Las interacciones entre analito y solvente son las que determinan la especificidad de la LC en fase reversa, ya que las interacciones del analito con la fase estacionaria son relativamente débiles, interacciones de Van der Waals no específicas. La retención disminuye generalmente al aumentar la polaridad del analito.

La cromatografía líquida de ultra resolución, cuyas siglas en inglés son UHPLC (*Ultra-High Performance (or Pressure) Liquid Chromatography*), está siendo una de las técnicas de separación más utilizadas en los últimos años. Utilizando columnas con tamaños de partículas menores de 2 μm, se consigue una mayor resolución y sensibilidad, gracias a la obtención de picos cromatográficos más estrechos y de mayor altura. Generalmente, se utilizan columnas cortas (5 o 10 cm), reduciendo también de ese modo, el tiempo de análisis.

Los contaminantes emergentes suelen estar presentes en matrices medioambientales relativamente complejas como son las aguas, suelos, sedimentos, o aire. La necesidad de detectar estos compuestos a bajos niveles de concentración hace que la espectrometría de masas (*Mass Spectrometry*, MS) sea la técnica analítica elegida por su universalidad, junto a la elevada selectividad, especificidad y sensibilidad. Desde el descubrimiento de los iones cargados positivamente en 1886 por E. Goldstein, los avances de J.J. Thompson, y la construcción del primer espectrómetro de masas con enfoque de velocidad por F.W. Aston en 1919, la espectrometría de masas es una de las técnicas analíticas que ha experimentado un mayor grado de desarrollo.

El poder combinado de ambas técnicas, cromatografía líquida acoplada a espectrometría de masas (LC-MS), hace que este acoplamiento sea el elegido en la actualidad para el análisis de contaminantes emergentes en muestras medioambientales.

En general, el analizador de MS es capaz de distinguir los distintos analitos en función de su diferente relación masa/carga (*mass/charge ratio, m/z)*, incluso si se produce co-elución de los compuestos en el sistema de separación. La clave del éxito en las medidas mediante MS recae en gran parte en la capacidad de conseguir que los compuestos neutros se conviertan en iones moleculares o fragmentos en estado

gaseoso. La ionización de los analitos es de vital importancia, ya que, controlar, separar y dirigir iones cargados resulta eficiente y sencillo mediante la aplicación de campos eléctricos y magnéticos.

Conseguir una fuente de ionización sencilla y robusta en el acoplamiento LC-MS resultó de gran dificultad debido a los altos flujos (alrededor de 1 mL/min) y la baja volatilidad de las fases móviles que debían evaporarse sin interferir con el alto vacío necesario en el analizador de MS (normalmente entre 10^{-4} y 10^{-7} Torr). Fue a partir de los años 80 cuando las interfases a presión atmosférica (*Atmospheric Pressure Interfaces*, APIs) empezaron a destacar sobre las demás interfases, debido a su capacidad de analizar compuestos de elevado peso molecular y a su robustez al permitir ionizar un amplio abanico de analitos en matrices de diversa naturaleza. Estas interfases se caracterizan por la formación de iones a presión atmosférica fuera de la zona de alto vacío, evitando los problemas técnicos relacionados con la incompatibilidad líquido/alto vacío. Actualmente, las interfases de ionización por *electrospray* (ESI) y por ionización química (*Atmospheric Pressure Chemical Ionization*, APCI) son las más utilizadas en los instrumentos LC-MS.

En esta Tesis sólo se ha utilizado la fuente de ionización de electrospray, que es la más universal y la de mayor aplicación en análisis medioambiental. Cuando el acoplamiento LC-MS se lleva a cabo mediante este tipo de interfase, los analitos disueltos en la fase móvil pasan al detector MS a través de un capilar de acero inoxidable a presión atmosférica sometido a un alto voltaje (3-6 kV). Este alto voltaje dispersa la corriente de líquido, formándose gotas altamente cargadas (nebulización), que son desolvatadas a medida que pasan a través de la región a presión atmosférica de la fuente del espectrómetro de masas (aprox. 120 ^{o}C). La desolvatación es asistida por una corriente de gas caliente, generalmente nitrógeno.

A medida que se evapora el disolvente las gotas se hacen cada vez más pequeñas, hasta que las fuerzas repulsivas entre cargas son capaces de superar las fuerzas cohesivas de tensión superficial. Se produce, entonces, la rotura final de la gota originando iones en fase gaseosa. Los iones que se forman en ESI pueden ser de carga positiva o negativa; generalmente se presentan como iones en modo positivo las moléculas protonadas, los aductos de sodio, potasio o amonio; mientras que los iones más frecuentes en modo negativo son las moléculas desprotonadas o los aductos de formiato o acetato. Dichos iones, positivos o negativos, entran en el espectrómetro de masas a través de lentes focalizadoras.

El analizador es la parte del espectrómetro que, trabajando a presiones muy bajas, permite separar los iones en fase gas que se han formado en la fuente de ionización, en función de su relación masa/carga. Atendiendo al modo en que estos iones son aislados, se diferencias distintos tipos de analizadores: cuadrupolo (Q), triple quadrupolo (QqQ), trampa de iones (IT, LIT), Orbitrap, resonancia de ion ciclotrón (ICR), sector magnético, o tiempo de vuelo (TOF). De todos ellos, a continuación se explicarán con mayor detalle el triple cuadrupolo, Orbitrap y TOF, que son los utilizados en esta Tesis Doctoral.

Como se ha dicho anteriormente, el acoplamiento LC-MS ha sido reconocido como una poderosa herramienta para el análisis de mezclas de gran complejidad. No obstante, y a pesar de su alta sensibilidad y selectividad, los instrumentos LC/MS se ven limitados por la diferente eficiencia de la ionización de los analitos en solvente y muestra, según el tipo de matriz introducida. La supresión/exaltación de la señal debido a los componentes de la matriz que ingresan al espectrómetro de masas conjuntamente con los analitos, limita la robustez y exactitud, además de ser una fuente potencial de errores sistemáticos.

Para minimizar estos errores existen distintas alternativas que se basan en la compensación de la supresión/exaltación (afecta a la cuantificación) o en la eliminación de los interferentes (afecta también a la detección). Posibles aproximaciones para conseguir estos fines son el uso de patrones internos, calibrado en matriz, dilución de la muestra o del extracto de muestra, pre-tratamiento de muestra para eliminar los interferentes (*clean-up*), o aplicación del método de adiciones estándares. Estas estrategias se explicarán con más detalle en el apartado "1.5.4 Efecto matriz en métodos LC-MS".

1.5.2 Cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS)

En las técnicas de ionización suave, como ocurre en las interfases ESI, los espectros obtenidos suelen estar dominados por la molécula (des)protonada $[M-H]^-/[M+H]^+$. Generalmente, con este tipo de interfases se obtiene información íntegra de la masa del compuesto, debido a la poca fragmentación, lo que limita la información estructural necesaria para confirmar que el pico obtenido corresponde a un cierto analito y no a otro compuesto isobárico que podría estar presente en la misma muestra. Esta información se puede conseguir aumentando la especificidad de los análisis. Una solución sería combinar varios analizadores en el tiempo o en el espacio, dando lugar así a la espectrometría de masas en tándem (*Tandem Mass Spectrometry*, MS/MS).

Un analizador de triple cuadrupolo (QqQ) consiste en la combinación de dos analizadores cuadrupolares separados entre sí por un hexapolo (o por un dispositivo denominado T-Wave en los más modernos), que actúa como celda de colisión. El modo *Selected Reaction Monitoring* (SRM) se basa en la monitorización de una transición concreta. Así, en el primer cuadrupolo se aísla un ion con una *m/z*

determinada (ion precursor) que pasa a la celda de colisión, donde se fragmenta por colisión con moléculas de gas inerte (generalmente argón). Este proceso recibe el nombre de disociación inducida por colisión (*Collision Induced Dissociation,* CID). El ion precursor se fragmenta en función de la estructura del analito; y los iones resultantes (iones producto) llegan al tercer cuadrupolo, en el que se selecciona uno de ellos. Este modo de trabajo proporciona una elevada sensibilidad, ya que se consigue disminuir notablemente la relación señal-ruido (*Signal/Noise ratio*, S/N) al disminuir considerablemente el ruido químico. Además, dado que se está trabajando con transiciones específicas se aumenta también la selectividad.

Con todo ello, la LC-MS/MS ha ido sustituyendo a otras técnicas menos específicas y poderosas como pueden ser la detección fluorimétrica, electroquímica y la espectrometría de masas de una sola etapa. Uno de los inconvenientes que presenta es la necesidad de optimizar ciertos parámetros relacionados con la adquisición, tales como, la selección del ion precursor, la energía de colisión, los iones producto o el voltaje del spray y de las lentes. Estos afectan de forma muy significativa a la sensibilidad y selectividad del método, y no pueden ser calculados a partir de la composición elemental o de la estructura química del analito.

Los métodos multi-residuos son imprescindibles en el área de la salud pública, en la que el químico analítico debe enfrentarse a una gran variedad de analitos, como fármacos, drogas, pesticidas, metabolitos u otros contaminantes prohibidos. Este hecho hace necesario el desarrollo y puesta a punto de metodología analítica capaz de analizar cientos de compuestos simultáneamente. Los equipos modernos de LC-MS/MS constituyen una herramienta ideal para estos fines, tal como se demuestra en esta Tesis Doctoral.

1.5.3 Cromatografía líquida acoplada a espectrometría de masas de alta resolución (LC-HRMS)

La espectrometría de masas de alta resolución (*High Resolution Mass Spectrometry*, HRMS) es una excelente herramienta para el análisis de moléculas de bajo peso molecular, ya que aporta información, en masa exacta, de todos los analitos que entran en el espectrómetro.

Analizador Orbitrap

El Orbitrap es un analizador de masas descrito por primera vez en el año 2000 por Alexander Makarov. Se compone de un electrodo exterior en forma de barril y un electrodo coaxial interior con forma de huso; juntos forman un campo electrostático con la distribución potencial cuadro-logarítmica.

El analizador de masas está precedido de un dispositivo de inyección externa, el *C-shaped ion Trap* (C-Trap), que permite el atrapamiento de una población significativa de iones antes de introducirlos al analizador mediante pulsos cortos, para que cada relación *m*/*z* de la población forme un pulso de micro-segundos. Estos pequeños paquetes de iones se focalizan hacia el Orbitrap mediante el electrodo exterior.

En un Orbitrap, los iones son inyectados tangencialmente en un campo eléctrico entre los electrodos y quedan atrapados porque su atracción electrostática hacia el electrodo interior es contrarrestada por la fuerza centrífuga. Aquellos con una específica relación *m*/*z* se mueven en anillos que oscilan alrededor del huso central. La frecuencia de estas oscilaciones armónicas es independiente de la velocidad del ion y es inversamente proporcional a la raíz cuadrada de la relación *m*/*z*. La trampa puede ser utilizada como un analizador de masa. La corriente de iones atrapados se

detecta dinámicamente, y es convertida utilizando la transformada de Fourier (*Fourier Transform*, FT) en la frecuencia y posteriormente en espectros de masas.

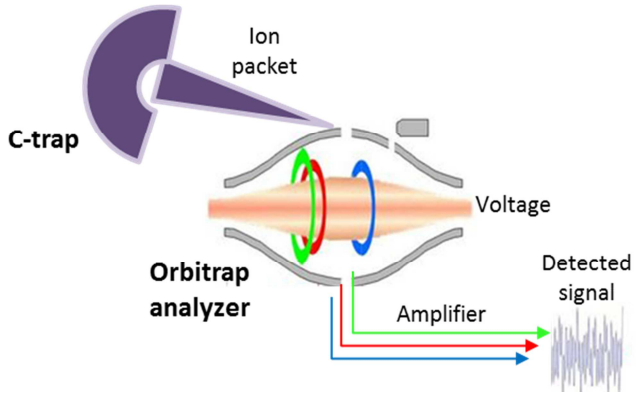


Figura 1.4 Esquema del acoplamiento C-Trap MS-Orbitrap MS.

El analizador Orbitrap tiene una alta precisión de masa (1-2 ppm), un alto poder de resolución (FWHM de hasta 400.000 en los equipos más modernos) y un aceptable rango dinámico.

Analizador tiempo de vuelo (TOF)

El analizador de tiempo de vuelo se basa en una separación de los iones en función del tiempo que tardan en atravesar un tubo de vuelo, el cual depende de la relación *m/z* (el tiempo de vuelo se encuentra entre 5-100 μs). Dado que todos los iones empiezan su viaje al mismo tiempo, o al menos en un intervalo de tiempo lo suficientemente corto, los iones más ligeros llegan al detector antes que los más pesados. Esto requiere que los iones que llegan continuamente procedentes de la interfase sean enviados eficientemente al TOF. Para una transmisión adecuada de los iones, se ha diseñado la aceleración ortogonal, que permite muestrear una fracción del haz de iones continuos (25% de los iones generados). Estos analizadores operan en modo discontinuo, ya que es necesario esperar a que todos los iones lleguen al detector, antes de volver a lanzar otro pulso. A pesar de esto, el porcentaje

de iones es mayor que el obtenido en un cuadrupolo operando en modo barrido total de los iones (*full scan*). Esto los convierte en uno de los instrumentos más sensibles en este modo de adquisición. El uso de reflectrones o espejos ópticos reenfoca sobre el detector los iones que tienen la misma *m*/*z*, consiguiendo aumentar la resolución, ya que disminuye la dispersión de velocidades iniciales favoreciendo que los iones con igual *m*/*z* alcancen el detector al mismo tiempo. Dicha resolución permite obtener medidas de masa exacta de los iones detectados.

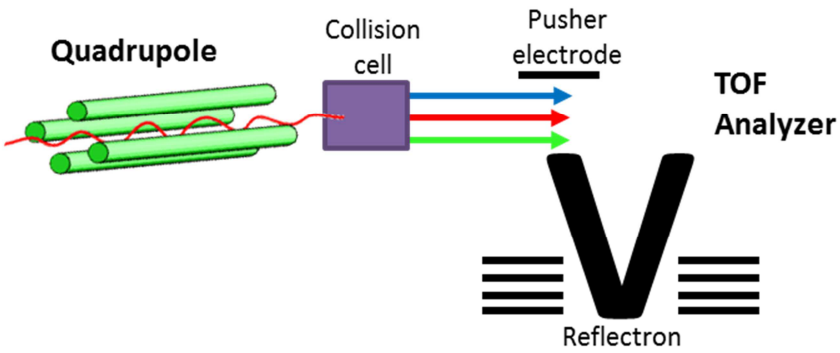


Figura 1.5 Cuadrupolo acoplado a un analizador de masas TOF.

Trabajar con masa exacta con un TOF, supone, aparte de la calibración diaria del equipo, una segunda y constante calibración del equipo. Esto se consigue mediante la adición de un compuesto de masa conocida (*lock mass*) a la entrada de la muestra, en la interfase del espectrómetro. La masa del analito se corrige posteriormente en función de la masa del lock mass, obteniéndose generalmente errores de masa inferiores a 5 ppm, lo cual suele facilitar la asignación de una fórmula molecular concreta para cada ion.

En esta Tesis se ha hecho uso de un analizador Orbitrap (durante la estancia realizada en *Mario Negri Institute* de Milán), y en mayor medida, de un analizador híbrido QTOF (*hybrid Quadrupole Time Of Flight*), que combina un analizador cuadrupolar con un analizador de tiempo de vuelo. La posibilidad de obtener el

espectro de iones fragmento con masa exacta permite identificar de manera absolutamente fiable los compuestos presentes en una muestra.

Además, tanto el Orbitrap como el QTOF ofrecen la posibilidad de investigar la presencia de un compuesto tras haber realizado el análisis y adquirido los datos, es decir, realizar un análisis retrospectivo. Esto es posible, ya que ambos analizadores trabajan en modo de espectro completo (*full spectrum acquisition, full scan*), que contiene información de toda la muestra, sin necesidad de pre-seleccionar iones antes de efectuar el análisis.

1.5.4 Efecto matiz en métodos LC-MS

El efecto matriz (*Matrix Effect*, ME) se puede definir como un aumento o disminución no esperada de la respuesta de los analitos, que se produce por la co-elución de otros componentes presentes en la matriz de la muestra. Aunque no se conoce el mecanismo exacto que lo origina, posiblemente tiene su origen en la competición entre los componentes no volátiles de la matriz y los iones de los analitos al acceder a la superficie de las gotas y pasar al estado gaseoso. Este hecho tendría lugar en la fuente de ionización y justificaría por qué se ha observado que el ME es un problema más acusado en la interfase ESI que en otras interfases (Yaroshenko, 2014).

Dependiendo de la matriz en cuestión y de las características físico-químicas de cada compuesto, así como del ambiente en el cual tiene lugar la ionización, la competencia de los componentes de la matriz puede provocar una disminución (denominada supresión de la señal) o un aumento (exaltación de la señal) de la eficiencia de la ionización de los analitos (Taylor, 2005).

El efecto matriz puede llegar a ser muy importante en algunos casos, y afectar a la selectividad, reproducibilidad, exactitud, linealidad y límite de cuantificación del método (Trufelli, 2011). Todo ello conllevaría importantes errores en la cuantificación (Gosetti, 2010).

Por ello, es de vital importancia estudiar el efecto matriz en cada una de las matrices de interés para los compuestos seleccionados. Éste es un proceso relativamente sencillo, siempre y cuando se disponga de matriz blanco de las mismas características que la muestra que se desea analizar. Para ello, se comparan las respuestas entre una disolución del patrón de referencia en solvente y el extracto blanco de muestra fortificado con el patrón, preferiblemente a la misma concentración. Se comparan las señales en ambos casos; cuando la respuesta del analito en extracto de muestra es menor que en disolvente, existe supresión de la ionización, en cambio, si es superior se produce una exaltación.

La corrección del efecto matriz en muestras de agua resulta complicada, especialmente en las matrices más complejas, como pueden ser aguas residuales urbanas. Entre las distintas estrategias dirigidas a eliminarlo, reducirlo o corregirlo, 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, la mejora de las condiciones de separación cromatográficas para evitar la co-elución de analitos e interferentes, la aplicación de calibrado en matriz, la dilución de la muestra, la aplicación de la metodología de adiciones estándar y el uso de patrones internos (Yaroshenko, 2014; Trufelli, 2011).

Mediante calibrado en matriz, usando extractos blanco de matriz, se consigue que la señal de patrones y muestras se vea afectada de modo similar por los interferentes de la matriz. La limitación de esta técnica es la complicada disponibilidad de un

blanco homogéneo de características similares a las de las muestras. Dicho inconveniente es prácticamente insalvable en el análisis de muestras como aguas de ríos o aguas residuales, en las que la composición de la muestra varía continuamente y no es fácil encontrar una muestra blanco que sea representativa del conjunto que se quiere analizar.

En el método de adiciones estándar se añaden cantidades crecientes de analito a una cantidad fija de muestra. Este método se ve limitado principalmente por dos aspectos: (a) Se requiere un análisis previo de la muestra para estimar la concentración del analito y poder ajustar las adiciones correctas. (b) Un aumento considerable del tiempo de análisis, ya que se necesitarán varias inyecciones por muestra.

La modificación del proceso de tratamiento de muestra, aplicando procesos más selectivos de extracción y/o aumentado la limpieza de las muestras (*clean-up*) tras la extracción, es otra alternativa para reducir el efecto matriz. Sin embargo, dicha aproximación tiene las principales desventajas del aumento de tiempo de análisis y de manipulación de la muestra, así como también la posibilidad de pre-concentrar ciertos interferentes estructuralmente semejantes al analito, pudiendo llegar a generar el efecto contrario al buscado.

El método de corrección más utilizado en el análisis de fármacos es, sin duda, el uso de patrones internos (Wong, 2009; Wille, 2012). Se considera patrón interno (*Internal Standard*, IS) de un analito, a aquel compuesto que presente unas características físico-químicas muy similares a las del mismo, es decir, que tengan similar retención en la LC, ionización en la interfase y fragmentación en la celda de colisión, pero que al mismo tiempo no pueda encontrarse presente en las muestras. Normalmente, las estructuras químicas de analito e IS suelen ser análogas. En principio, el patrón ideal

para corregir el ME es el mismo analito marcado isotópicamente. Los patrones marcados con ^{13}C y deuterio (D) son los más utilizados, pues su abundancia en la naturaleza es extremadamente baja (entre el 1 y 0.015%) y por lo tanto, no interfieren en la cuantificación.

El procedimiento analítico habitual consiste en añadir una cantidad conocida de patrón interno al principio del proceso de extracción (*surrogate*), ya que, de este modo se corrigen las posibles pérdidas en el pre-tratamiento de muestra y también las variaciones derivadas del efecto matriz. La corrección se basa en que los iones de los analitos y de sus análogos marcados son químicamente equivalentes y la matriz les afecta de un modo similar (Castiglioni, 2006).

En el caso de los métodos multi-residuales, resulta prácticamente imposible corregir cada analito con su propio compuesto marcado, especialmente en aquellos casos en los que se incluye un elevado número de compuestos en el método. Las razones de esta dificultad estriban en la limitada disponibilidad comercial de patrones marcados, así como en su elevado precio. En el caso de no disponer del propio analito marcado, la alternativa más utilizada consiste en efectuar la corrección con otro patrón interno análogo. Su selección se basa en la similitud entre las estructuras químicas, o en su defecto, en función del tiempo de retención de ambos. Sin embargo, el uso de patrones internos análogos no siempre asegura una satisfactoria corrección del efecto matriz (Marín, 2009).

Otra alternativa sencilla de minimizar el ME es la dilución del extracto de la muestra. Reducir la presencia de otros componentes de la matriz mediante una adecuada dilución en el extracto puede reconducir a que la respuesta de los analitos en la matriz sea comparable a los patrones en solvente. Dicha dilución de la matriz puede presentar otras ventajas como son: una mejor forma de pico, mayor

estabilidad del tiempo de retención y mejor mantenimiento de la columna cromatográfica. No obstante, el principal inconveniente de dicha aproximación es la pérdida de sensibilidad inherente a la dilución, desaconsejándose cuando la sensibilidad requerida sea máxima. En este sentido, la sensibilidad del equipo instrumental utilizado es fundamental, por lo que son los equipos de última generación los que permiten usar esta aproximación en condiciones más ventajosas.

CAPÍTULO 2

DESARROLLO DE METODOLOGÍA ANALÍTICA MULTI-RESIDUAL BASADA EN LC-MS/MS PARA LA DETERMINACIÓN DE FÁRMACOS Y DROGAS DE ABUSO EN AGUAS

2.1 Introducción

2.2 Artículo Científico 1: *Fast determination of 40 drugs in water using large volume direct injection liquid chromatography–tandem mass spectrometry.* Talanta 131 (2015) 719-727

2.3 Discusión de los resultados

- 2.3.1 Optimización de las condiciones MS/MS
- 2.3.2 Optimización cromatográfica
- 2.3.3 Validación del método
- 2.3.4 Análisis de muestras reales

2.1 Introducción

La investigación de fármacos y drogas de abuso en el medio ambiente es un campo de estudio reciente. Trabajos realizados previamente centran su interés en los compuestos más consumidos (Baker, 2013), catalogados por la cantidad de unidades dispensadas por las farmacéuticas o por la presencia, reportada en artículos científicos, de dichos compuestos en el medio ambiente.

El trabajo que se presenta en este capítulo surgió a raíz de una colaboración con el laboratorio de análisis medioambiental IPROMA S.L., con el objetivo común de desarrollar métodos rápidos y sensibles para la detección de compuestos emergentes presentes en aguas. Aprovechando los equipos analíticos disponibles en cada laboratorio, se desarrollaron dos metodologías analíticas por separado. La primera se basaba en una extracción SPE *on-line* combinada con la separación de los analitos mediante HPLC y la detección por MS/MS. Éste era el procedimiento aplicado hasta el momento de la colaboración por el mencionado laboratorio. La segunda consistía en una introducción directa de la muestra, sin pre-tratamiento, en el equipo UHPLC-MS/MS. Esta última es la que se estudió en esta Tesis Doctoral y

se detalla en el presente capítulo, en el cual se aborda la optimización y validación para la determinación de fármacos y drogas en aguas mediante inyección directa de las muestras. Una vez validadas satisfactoriamente ambas metodologías, se aplicaron a las mismas muestras de agua, 10 superficiales y 10 residuales, con el fin de comparar los resultados y comprobar su robustez.

Se seleccionaron inicialmente 8 de los fármacos más consumidos en España, según datos oficiales proporcionados por el Ministerio de Sanidad Español (IT, 04/2011). 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*). Tras una amplia revisión bibliográfica, se decidió incluir también otros fármacos, que, aunque no estaban catalogados dentro de las listas de los más consumidos, habían sido comúnmente detectados en aguas (Fatta-Kassinos, 2011a; Gracia-Lor, 2011). Cabe mencionar que muchos de estos compuestos pueden adquirirse sin receta médica, por lo que resulta comprensible su elevada frecuencia de detección en el medio ambiente. Así se elaboró una lista final de 35 fármacos, a la que se añadieron 5 compuestos más, correspondientes a las drogas de abuso más consumidas según diversos estudios recientemente publicados (cocaína, benzoilecgonina, cocaetileno, anfetamina y MDMA) (Castiglioni, 2013; Pal, 2013; Thomas, 2012). La selección de los compuestos en base a los criterios señalados nos permitió tener una visión más realista de la presencia de los fármacos y las drogas en el medio ambiente acuático.

Tal y como afirman autores como Seifrtová (2009) o Buchberger (2011), en el futuro, no se requerirá una etapa de tratamiento de muestra. La inyección directa al sistema de detección será la tendencia general, que se irá reafirmando a medida que se desarrollen equipos cada vez más sensibles. La inyección directa conlleva

importantes ventajas como son: reducción del tiempo de análisis (ya que no hay que destinar tiempo ni esfuerzos al proceso de extracción), menor riesgo de contaminación o pérdida de muestra y disminución de costes, tanto de solventes y reactivos como salarial. Sin embargo, este proceso entraña una cierta dificultad relacionada con el efecto matriz. Normalmente, se requiere la inyección de grandes volúmenes para alcanzar los límites de detección y cuantificación exigidos, al no existir una etapa de pre-concentración; y ello implica que gran parte de muestra entra al sistema LC-MS pudiendo afectar de diversas maneras, según la composición de la matriz. Por ello, muchas veces resulta complicado obtener recuperaciones satisfactorias para todas las aguas, debido al fuerte efecto matriz, precisando de compuestos marcados isotópicamente para corregir sus respuestas.

En el trabajo que se presenta a continuación, se ha hecho uso de la cromatografía líquida de ultra resolución. Para ello se han utilizado columnas de BEH (*Ethylene Bridged Hybrid*) con partícula híbrida (de tamaño de 1.7 μm) que combina las propiedades de los rellenos inorgánico (sílice) y orgánico (polimérico). La fase estacionaria enlaza las cadenas hidrocarbonadas de C18 que se unen a la sílice mediante puentes de etileno. El uso de estas columnas proporciona mayor resolución cromatográfica y un aumento de la sensibilidad mediante la obtención de picos cromatográficos más estrechos (entre 5-10 s de anchura) y más definidos. Para poder aprovechar las ventajas que ofrecen las columnas de UHPLC se requieren velocidades lineales altas, es decir, flujos de fase móvil relativamente elevados. Esto genera elevadas presiones en el sistema, por lo que se precisa de una instrumentación avanzada capaz de trabajar a altas presiones.

Los analizadores de triple cuadrupolo son una herramienta muy poderosa, y por ello, ampliamente utilizados para análisis cuantitativo. El modo de trabajo SRM, por

su elevada sensibilidad y mejora de la relación S/N, posibilita la determinación de diferentes contaminantes orgánicos en aguas a niveles muy bajos de concentración. Trabajando en SRM, los analitos se deben seleccionar previamente a la inyección en el sistema LC-MS (métodos *pre-target*). Su principal inconveniente es la falta de capacidad para detectar otros compuestos presentes en una muestra que no hayan sido seleccionados e incluidos en el método, aunque estos se encuentren a elevados niveles de concentración. El modo *target* implica la optimización de las condiciones MS/MS de cada compuesto, estableciendo en primer lugar el modo de ionización y el voltaje de cono para definir el ion precursor, así como 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 la selectividad de la transición.

El criterio de confirmación que se sigue en la mayoría de los métodos desarrollados para muestras medioambientales se basa en la Decisión 2002/657/EC de la Comisión Europea, en la que se proponen unas pautas para la cuantificación y confirmación de contaminantes y residuos orgánicos en muestras de alimentos de origen animal. Debido a la ausencia de directrices en el campo medioambiental, directrices como ésta, o como la guía SANCO para análisis de residuos (SANCO, 2013), en los últimos años se han aplicado también en este campo de trabajo.

Ambas directrices establecen que para poder identificar correctamente un compuesto en una muestra se han de monitorizar al menos dos transiciones en modo tándem MS, o dos iones en HRMS, y además se ha de cumplir la relación de intensidad (*ion ratio,* q/Q) entre las transiciones seleccionadas. Esta relación debe ser semejante a la obtenida para un patrón, de acuerdo con unas tolerancias

establecidas en función de su intensidad relativa **(Tabla 2.1).** Asimismo, el tiempo de retención entre el compuesto y el patrón no debe desviarse más del 2.5%.

Tabla 2.1 Tolerancias máximas permitidas para la confirmación de contaminantes.

Ion ratio (least/most intense ion)	Maximum tolerance (relative) for $LC\text{-}MS^n$, LC-MS, $GC\text{-}MS^n$, GC-CI-MS	
	SANCO 2013	Decisión 2002/657/EC
0.50-1.00	± 30%	± 20%
0.20-0.50	± 30%	± 25%
0.10-0.20	± 30%	± 30%
<0.10	± 30%	± 50%

En el trabajo que se presenta a continuación se desarrolló un método analítico para la determinación de 35 fármacos y 5 drogas de abuso frecuentemente consumidas en España. La metodología desarrollada evita el tratamiento de muestra, basándose en una inyección directa de la muestra de agua, superficial (SW) o residual (efluente urbano, EWW), seguida de cromatografía líquida (UHPLC) acoplada a un analizador de triple cuadrupolo de última generación (Xevo TQS, Waters Micromass, Manchester, UK).

2.2 Artículo Científico 1

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Fast determination of 40 drugs in water using large volume direct injection liquid chromatography–tandem mass spectrometry

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ABSTRACT

This work describes a rapid analytical method based on direct sample injection of water samples for the simultaneous identification/quantification of 40 emerging compounds, including pharmaceuticals and drugs of abuse. The water samples were analyzed by ultra-high-performance liquid chromatography coupled to hybrid triple quadrupole mass spectrometer (UHPLC–MS/MS QqQ). Taking profit of the increasing sensitivity of nowadays tandem mass spectrometers, direct sample injection of large volumes has been an attractive alternative to pre-concentration steps. In this work, the developed methodology has been validated at three concentration levels (10, 100 and 1000 ng/L) in 10 different water samples of different types (5 effluent wastewater and 5 surface water samples). The majority of compounds could be satisfactory validated at these concentrations, showing good recoveries and precision. With only few exceptions, the limits of quantification (LOQs), estimated from the sample chromatogram at lowest spiked level tested, were below 3 ng/L. The method was applied to the analysis of 10 effluent wastewater and 10 surface water samples. Venlafaxine was the compound most frequently detected (80%) in surface water, followed by acetaminophen (70%). Regarding effluent wastewater, valsartan and 4-acetyl aminoantipyrine were detected in 9 out of 10 samples analyzed. These two compounds together with 4-formyl aminoantipyrine and naproxen showed the highest concentrations (> 2000 ng/L). In these cases, a dilution step was required for a correct quantification. As an additional evaluation of the method performance, the same water samples were analyzed in another laboratory by a second analytical methodology, based on on-line solid-phase-extraction coupled to LC–MS/MS (QqQ).

1. Introduction

The presence of human and veterinarian pharmaceuticals, as well as illegal drugs of abuse, in environmental samples has been recognized as a potential environmental threat [1,2]. These groups of contaminants are of present concern, due to their very high biological activity, psychoactive properties and still not well known effects to the aquatic environment [1,3]. After their consumption, these compounds can be excreted as the parent compound, as metabolites or as a mix of unchanged compound plus metabolites, reaching first the wastewater treatment plants (WWTPs) and finally the aquatic environment if they are not completely removed by WWTPs. The concentrations of these compounds in the environment depend on many factors, including their consumption pattern and use, the percentage of wastewater collected and the characteristics of the processes used for wastewater treatment [4]. Recently, several works have reported the presence of drugs and metabolites in the environmental, showing concern for its unknown impact [5–7].

Current analytical methods developed for quantifying low concentration of pharmaceuticals [2,8–10] and illicit drugs [11,12] in aquatic samples, usually include pre-concentration steps, the most common being those based on solid-phase extraction (SPE). Extraction from water samples has usually been performed by off-line SPE [5,6,8,9,11], although on-line SPE-LC has also been reported as a time and cost-saving alternative thanks to its fully automation [7,13]. Large-volume injection (LVI) is an attractive approach for aqueous samples that has been applied in several works as a rapid and efficient alternative to conventional SPE [14–18]. Typically, LVI involves the direct injection of sample volumes that range from 100 to 5000 μL versus the more conventionally injected volumes of 10–20 μL [14]. The improvement in sensitivity comes from the injection of sample volumes larger than usual. LVI provides good reproducibility and low sample contamination as a consequence of

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FAST DETERMINATION OF 40 DRUGS IN WATER USING LARGE VOLUME DIRECT INJECTION LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Talanta, 131 (2015) 719–727

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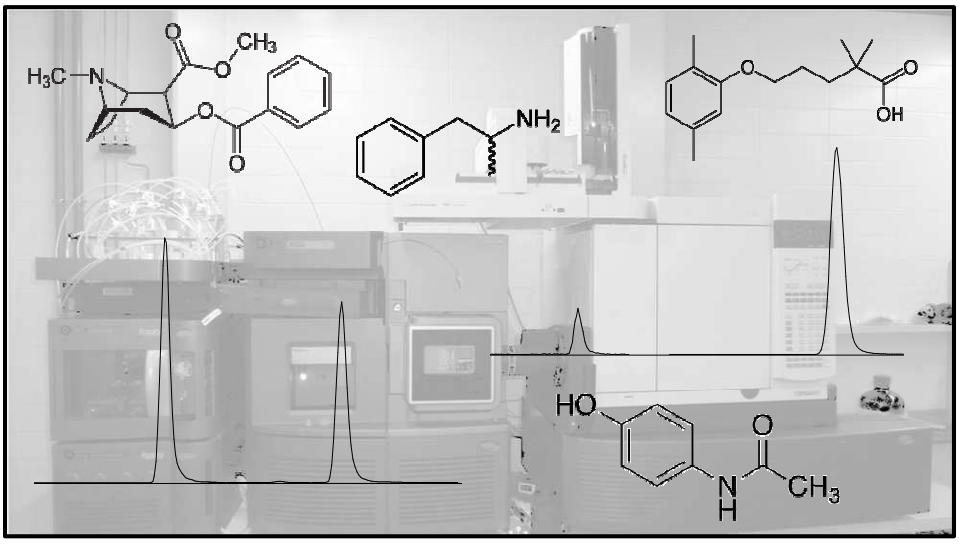
Graphical Abstract



Highlights

- Rapid and sensitive analytical methodology developed for the analysis of 40 drugs in water.
- Methodology based on direct sample injection and UHPLC–MS/MS QqQ.
- Quantitative validation performed in SW and EWW at three concentration levels.
- Analysis of 10 EWW and 10 SW showed up to 32 positive findings.
- Water samples re-analyzed by on-line SPE-LC–MS/MS (QqQ) in an accredited laboratory.

ABSTRACT

This work describes a rapid analytical method based on direct sample injection of water samples for the simultaneous identification/quantification of 40 emerging compounds, including pharmaceuticals and drugs of abuse. The water samples were analyzed by ultra-high-performance liquid chromatography coupled to hybrid triple quadrupole mass spectrometer (UHPLC–MS/MS QqQ). Taking profit of the increasing sensitivity of nowadays tandem mass spectrometers, direct sample injection of large volumes has been an attractive alternative to pre-concentration steps. In this work, the developed methodology has been validated at three concentration levels (10, 100 and 1000 ng/L) in 10 different water samples of different types (5 effluent wastewater and 5 surface water samples). The majority of compounds could be satisfactory validated at these concentrations, showing good recoveries and precision. With only few exceptions, the limits of quantification (LOQs), estimated from the sample chromatogram at lowest spiked level tested, were below 3 ng/L. The method was applied to the analysis of 10 effluent wastewater and 10 surface water samples. Venlafaxine was the compound most frequently detected (80%) in surface water, followed by acetaminophen (70%). Regarding effluent wastewater, valsartan and 4-acetyl aminoantipyrine were detected in 9 out of 10 samples analyzed. These two compounds together with 4-formyl aminoantipyrine and naproxen showed the highest concentrations (>2000 ng/L). In these cases, a dilution step was required for a correct quantification. As an additional evaluation of the method performance, the same water samples were analyzed in another laboratory by a second analytical methodology, based on on-line solid-phase-extraction coupled to LC–MS/MS (QqQ).

Keywords

Direct injection, illicit drugs, pharmaceuticals, effluent wastewater, surface water, liquid chromatography, triple quadrupole mass spectrometry.

1. INTRODUCTION

The presence of human and veterinarian pharmaceuticals, as well as illegal drugs of abuse, in environmental samples has been recognized as a potential environmental threat [1] and [2]. These groups of contaminants are of present concern, due to their very high biological activity, psychoactive properties and still not well known effects to the aquatic environment [1] and [3]. After their consumption, these compounds can be excreted as the parent compound, as metabolites or as a mix of unchanged compound plus metabolites, reaching first the wastewater treatment plants (WWTPs) and finally the aquatic environment if they are not completely removed by WWTPs. The concentrations of these compounds in the environment depend on many factors, including their consumption pattern and use, the percentage of wastewater collected and the characteristics of the processes used for wastewater treatment [4]. Recently, several works have reported the presence of drugs and metabolites in the environmental, showing concern for its unknown impact [5], [6] and [7].

Current analytical methods developed for quantifying low concentration of pharmaceuticals [2], [8], [9] and [10] and illicit drugs [11] and [12] in aquatic samples, usually include pre-concentration steps, the most common being those based on solid-phase extraction (SPE). Extraction from water samples has usually been performed by off-line SPE [5], [6], [8], [9] and [11], although on-line SPE-LC has also been reported as a time and cost-saving alternative thanks to its fully automation [7] and [13]. Large-volume injection (LVI) is an attractive approach for aqueous samples that has been applied in several works as a rapid and efficient alternative to conventional SPE [14], [15], [16], [17] and [18]. Typically, LVI involves the direct injection of sample volumes that range from 100 to 5000 µL versus the more conventionally injected volumes of 10–20 µL [14]. The improvement in sensitivity comes from the injection of sample volumes larger than usual. LVI provides good reproducibility and low sample contamination as a consequence of the minimal sample handling. Moreover, it allows to increase sample throughput at minimal cost compared to both off- and on-line SPE, because no SPE cartridges and solvents are needed [14]. Despite the injection of larger volumes, modern and sensitive instruments

are commonly needed for final measurement, as the increase in injection volume does not compensate the pre-concentration factors normally reached by SPE. In addition, peak shape may be deteriorated for early eluting analytes when increasing injection volume despite the lower eluotropic strength of water sample. Moreover, only clean water is usually directly injected in the system otherwise matrix effects could not be properly compensated for. Although, we show that effluent wastewater might be considered clean water in our LVI approach.

Modern multi-class methods applied for the determination of polar pharmaceuticals or drugs of abuse are mostly based on liquid chromatography (LC). The use of UHPLC in combination with tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) [2], [8], [10], [11], [19], [20], [21] and [22] or ion trap (IT) analyzers [23], [24], [25], [26] and [27], has made possible the development of faster and more sensitive methods. Moreover, the fact of working with short dwell times in new instruments, allows increasing the number of selected reaction monitoring (SRM) transitions acquired simultaneously per compound making possible not only quantification but also a reliable identification. Although LC–MS/MS is the technique of choice at present to analyze polar compounds in aquatic samples, the presence of pharmaceuticals in environmental samples has also been investigated by LC coupled to high resolution mass spectrometry (HRMS), using time-of-flight (TOF MS) [28], [29] and [30] or Orbitrap analyzers [31], [32] and [33]. HRMS analyzers have strong potential for large screening and for identification/elucidation purposes, but they show less sensitivity than state-of-the-art MS/MS instruments, making that LC–MS/MS are considered the optimum analyzers for quantification at trace level.

The goal of the present paper is to develop fast and sensitive analytical methodology combining the advantages of UHPLC–MS/MS with last-generation triple quadrupole and large-volume direct sample injection. Thus, a rapid method avoiding sample manipulation (i.e. pre-concentration and clean-up) has been developed for the determination of forty highly consumed compounds, including pharmaceuticals, drugs of abuse and some veterinary drugs, in water samples. The quantitative validation has been performed at three concentration levels (10, 100 and 1000 ng/L) in 5 surface water (SW)

and 5 effluent wastewater (EWW) samples. Several isotopically-labeled internal standards have been tested for correction of expected matrix effects. In order to evaluate the applicability of the method, 20 water samples (10 SW and 10 EWW) were analyzed. The same samples were analyzed by another laboratory using a methodology based on on-line SPE-LC–MS/MS (QqQ).

2. EXPERIMENTAL

2.1. Reagents and chemicals

Pharmaceutical 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). All reference standards presented purity higher than 93%.

Illicit drugs and metabolites studied were amphetamine, 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), cocaine, cocaethylene and benzoylecgonine. These compounds were obtained from Sigma-Aldrich (Madrid, Spain), Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol, acetonitrile or as salt.

Standard stock solutions of each compound were prepared at 100 mg/L in methanol or acetonitrile. Intermediate solutions (10 mg/L) were prepared by dilution of the stock solution ten-fold with methanol. Mixed working solutions containing all analytes were prepared daily from intermediate solutions by appropriate dilution with water, and were used for preparation of the aqueous calibration standards and for spiking samples in the validation study.

Isotopically-labeled internal standards (ILIS) of omeprazole-d_3, acetaminophen-d_4, diclofenac-d_4, valsartan-d_8, carbamazepine 10,11-epoxide-d_{10} and salicylic acid-d_3 were from CDN Isotopes (Quebec, Canada); atorvastatin-d_5 from Toronto Research Chemicals and sulfamethoxazole-$^{13}C_6$ and trimethoprim-$^{13}C_3$ were from Cambridge Isotope Laboratories (Andover, MA, USA). Deuterated drugs of abuse were purchased from Cerilliant as solutions in methanol or acetonitrile at a concentration of 100 mg/L (amphetamine-d_6, MDMA-d_5, benzoylecgonine-d_3, cocaine-d_3 and cocaethylene-d_8). A mix ILIS working solution at 100 µg/L was prepared in MeOH and used as internal standard. All solutions were stored in amber glass bottles at −20 °C.

HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), formic acid (HCOOH, content >98%), ammonium acetate (NH_4Ac, reagent grade) and sodium hydroxide (NaOH, >99%) 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).

2.2. Instrumentation

UHPLC analysis were carried out with a Waters Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA), equipped with a binary solvent manager and a sample manager. Chromatography separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size analytical column 100 mm×2.1 mm (Waters). The mobile phases used were A=H_2O and B=MeOH, both with 0.01% HCOOH and 1 mM NH_4Ac. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 5%; 7 min, 90%; 8 min, 90%; 8.1 min, 5%; 10 min, 5%. The flow rate was 0.4 mL/min. The column was kept at 40 °C and the sample manager was maintained at 5 °C. Analysis run time was 10 min. The sample injection volume was 100 µL.

A Waters Acquity UPLC system was interfaced to a triple quadrupole mass spectrometer Xevo TQS (Waters) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operated in positive and negative ion mode. Cone gas as well as desolvation gas was nitrogen (Praxair, Valencia, Spain) set up 250 L/h and 1200 L/h, respectively. For operation in the MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) with a pressure of 4×10^{-3} mbar in the collision cell (0.15 mL/min). Other parameters optimized were capillary voltages 3.5 kV (ESI+) and 3.0 kV (ESI–); source temperature 150 °C and desolvation temperature 650 °C. Cone voltage was selected as 10 V for all compounds, due to no variations were observed. Dwell times were automatically selected in order to obtain enough points per peak and can be decreased down to 3 ms.

All data were acquired and processed using MassLynx v 4.1 software (Waters).

2.3. Sample preparation

All water samples were centrifuged at 4500 rpm for 5 min. 1-mL surface water or effluent wastewater was spiked at 50 ng/L with the ILIS mix. 100 µL of the sample was directly injected in the UHPLC–MS/MS system.

2.4. Validation study

Acquisition was performed in SRM mode, with the (de)protonated molecular ion of each compound chosen as precursor ion. The most abundant product ion of each target analyte was typically used for quantification and two additional product ions were used for confirmation. LC retention time was also compared with that of the reference standards (within ±2.5%) to help to confirm the compounds detected in samples. 14 compounds were quantified using their corresponding labeled analyte as internal standard and 5 compounds were quantified using an analog IS (see Table 2). The remaining 21 compounds were quantified by external calibration using absolute responses.

The linearity of the method was studied by analyzing standard solutions in triplicate at eight concentrations, in the range from 1 to 2500 ng/L. Satisfactory linearity was assumed when the correlation coefficient (r^2) was higher than 0.99, based on relative responses (analyte peak area/ILIS peak area), except for those compounds that were quantified without ILIS (absolute response).

Method accuracy (estimated by means of analysis of spiked samples directly injected into the LC–MS/MS system) and precision (expressed as repeatability, in terms of relative standard deviation (RSD)) were evaluated in surface water and effluent wastewater, spiked at three concentrations (10, 100 and 1000 ng/L). A total of 10 different water samples were used for the method validation (5 effluent wastewater and 5 surface water samples). Quantification was made by using calibration standards in solvent and relative or absolute responses as a function of the ILIS was used or not for matrix effects correction. 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. Adequate blank samples were not found for several analytes as they were present in all samples collected. In these cases, LOQ values were estimated from the chromatograms of the non-spiked "blank" samples, considering the concentration levels.

2.5. Water samples

20 water samples (10 EWWs and 10 SWs) were collected in polyethylene high-density bottles in selected sites of the Spanish Mediterranean area (Castellon and Valencia provinces). Composite EWW samples were collected from different WWTPs using primary and secondary treatment methods. Grab SW were sampled from different rivers (3), reservoirs (3) and lakes (4). All samples were taken from October to December in 2012. Samples were stored at −18 °C until analysis. Before analysis, samples were thawed at room temperature.

2.6. On-line SPE LC-MS/MS QqQ

An alternative analytical methodology was also applied following the protocol used in routine analysis by an ISO 17025 certified laboratory in Spain (IPROMA S.L.). For LC analysis, an Agilent 1200SL binary pump was coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer system API3200QTRAP from Applied Biosystems–Sciex (Foster City, California, USA). On-line SPE was performed by using an Agilent 1200 pump and a Strata-X cartridge (2×20 mm, 25 µm) from Phenomenex (Torrance, CA, USA). This equipment also includes a PAL autosampler (CTC Analytics, Switzerland) for automated sample injection. The injection volume to the on-line SPE was 2 mL of water, previously centrifuged at 2500 rpm for 5 min (wastewater samples were diluted 1/20). Chromatographic separation was performed on a reversed-phase column ZORBAX Eclipse XDB-C18 (50×4.6 mm, 1.8 µm) from Agilent (Palo Alto, CA, USA) maintained at 40 °C. Mobile phases A and B were 0.1% formic acid in water and 0.1% formic acid in methanol, respectively. The following linear gradient was used: hold at 95%A for 4.5 min, decreased to 70%A over 4.6 min, decreased to 0%A over 6.5 min and then increased to 95%A over 10.1 min, returning to the initial conditions. The flow rate was set to 600 µL/min [34].

3. RESULTS AND DISCUSSION

In this work, 35 human and veterinary pharmaceuticals and 5 drugs of abuse were selected (Table 1). Eight pharmaceuticals were among the most widely consumed in Spain [35]. The rest of compounds were selected due to their reported presence in water samples and to their potential negative effect on living organisms of the aquatic environment. Moreover, 4 compounds corresponded to metabolites of pharmaceuticals: salicylic acid, metabolite of acetylsalicylic acid [36]; and 4-aminoantipyrine, 4-acetyl aminoantipyrine and 4-formyl aminoantipyrine, metabolites of dipyrone [37], [38] and [39].

3.1. MS/MS optimization

Individual standard solutions were directly infused in the MS/MS system. The majority of the compounds (33 out of 40) were determined with ESI operating in positive ionization mode, using the protonated molecule $[M+H]^+$ as precursor ion. The 7 remaining compounds were determined in negative ionization using $[M-H]^-$ as precursor ion. The three most sensitive SRM transitions (in terms of signal-to-noise ratio) were selected for each compound. The most abundant was used for quantification (Q) whereas the other two transitions were acquired for confirmation (q_1, q_2). The only exception was salicylic acid (only one transition), and gemfibrozil and naproxen (two transitions) because of their poor fragmentation. MS/MS parameters as well as SRM transitions and retention times are listed in Table 1. This table also shows the average (q/Q) ratios obtained from the calibration standards. The RSDs for q/Q ratios illustrate whether these ratios might be considered to be concentration dependent or not (e.g. RSD<15% would indicate little variation of the q/Q values over the concentration range tested, from 1 to 2500 ng/L).

Three SRM transitions were acquired per compound, whereas for ILIS, only the quantification transition was monitored. Using our fast-acquisition triple quadrupole mass analyzer, dwell times as low as 3 ms per transition could be automatically set up allowing satisfactory peak shape (at least 10 points-per peak) and sensitivity for all 40 compounds investigated.

Table 1. MS/MS optimized conditions for selected compounds.

Compound	ESI	T_R (min)	Precursor ion (m/z)	Q transition	C.E. (eV)	q_1 transition	C.E. (eV)	q_1/Q (RSD)	q_2 transition	C.E. (eV)	q_2/Q (RSD)
4-Acetyl aminoantipyrine	+	2.85	245.9	246 > 228	10	246 > 83	20	0.71(3)	246 > 104	20	0.38(4)
4-Aminoantipyrine	+	3.15	204.1	204 > 56	15	204 > 159	10	0.29(5)	204 > 83	15	<0.01(5)
4-Formyl aminoantipyrine	+	2.81	232.1	232 > 83	20	232 > 104	20	0.63(4)	232 > 214	10	0.63(7)
Acetaminophen	+	1.99	152.1	152 > 110	15	152 > 65	25	0.20(5)	152 > 93	20	0.26(8)
Alprazolam	+	5.77	308.9	309 > 281	25	309 > 205	25	0.11(4)	309 > 274	25	0.21(5)
Amphetamine	+	2.81	136.2	136 > 91	15	136 > 119	10	0.43(6)	136 > 65	10	0.12(15)
Atorvastatin	+	6.68	558.9	559 > 440	20	559 > 466	15	0.20(5)	559 > 292	25	0.17(4)
Benzoylecgonine	+	3.32	290.0	290 > 168	15	290 > 105	25	0.34(6)	290 > 92	25	<0.01(16)
Bezafibrate	-	6.10	359.8	360 > 274	20	360 > 154	25	0.24(5)	360 > 85	15	0.07(6)
Carbamazepine	+	5.32	236.9	237 > 194	20	237 > 192	20	0.24(5)	237 > 179	25	0.08(5)
Clarithromycin	+	6.11	590.0	590 > 158	20	590 > 116	25	0.20(13)	590 > 98	25	0.06(9)
Cocaethylene	+	4.24	318.0	318 > 196	20	318 > 82	25	0.71(4)	318 > 150	25	0.18(7)
Cocaine	+	3.74	304.1	304 > 182	15	304 > 82	25	0.56(8)	304 > 105	25	0.19(9)
Diclofenac	-	6.87	294.1	294 > 250	10	294 > 214	20	0.04(3)	294 > 178	20	<0.01(5)
Enalapril	+	4.99	376.9	377 > 234	15	377 > 117	25	0.24(4)	377 > 303	15	0.30(10)
Erythromycin	+	5.62	734.2	734 > 158	25	734 > 576	15	0.11(5)	734 > 558	15	0.03(10)
Florfenicol	-	3.32	355.7	356 > 336	10	356 > 185	20	1.00(1)	356 > 119	25	0.04(15)
Flumequine	+	5.11	261.9	262 > 244	15	262 > 202	25	0.30(9)	262 > 174	25	0.01(13)
Furaltadone	+	2.37	324.9	325 > 100	20	325 > 252	15	1.00(6)	325 > 281	10	0.77(4)
Gemfibrozil	-	7.46	248.9	249 > 121	20	249 > 127	10	0.07(10)			

Table 1 (Cont). MS/MS optimized conditions for selected compounds.

Compound	ESI	TR (min)	Precursor ion (m/z)	Q transition	C.E. (eV)	q1 transition	C.E. (eV)	q1/Q (RSD)	q2 transition	C.E. (eV)	q2/Q (RSD)
Irbesartan	+	6.26	428.8	429 > 207	25	429 > 195	20	0.17(2)	429 > 180	25	0.04(4)
Levamisol	+	2.48	205.0	205 > 178	20	205 > 91	25	0.29(10)	205 > 123	25	0.43(11)
Lincomycin	+	2.89	407.0	407 > 126	20	407 > 359	15	0.07(9)	407 > 389	15	0.03(9)
Lorazepam	+	5.76	320.9	321 > 275	20	321 > 303	15	0.50(6)	321 > 229	25	0.34(8)
MDMA	+	2.90	194.0	194 > 163	10	194 > 105	20	0.34(5)	194 > 135	20	0.33(9)
Nalidixic acid	+	4.92	233.0	233 > 215	10	233 > 187	25	0.71(4)	233 > 159	25	0.19(8)
Naproxen	-	6.11	230.2	185 > 169	20	229 > 169	15	0.01(15)			
Olanzapine	+	3.25	312.9	313 > 256	20	313 > 84	20	0.56(12)	313 > 213	25	0.45(10)
Omeprazole	+	5.23	345.7	346 > 198	10	346 > 136	25	0.45(3)	346 > 151	15	0.32(4)
Oxolinic acid	+	4.24	261.9	262 > 244	15	262 > 216	25	0.13(12)	262 > 158	25	0.04(8)
Pantoprazole	+	5.18	383.9	384 > 200	10	384 > 138	25	1.10(4)	384 > 153	15	0.36(5)
Pravastatin	-	5.76	423.0	423 > 321	15	423 > 303	15	1.00(5)	423 > 101	25	0.53(11)
Roxithromycin	+	6.22	679.1	679 > 158	25	679 > 116	25	0.22(6)	679 > 98	25	0.04(16)
Salicylic acid	-	4.26	137.0	137 > 93	15						
Sulfadiazine	+	2.11	251.0	251 > 156	15	251 > 92	25	0.71(5)	251 > 108	20	0.43(2)
Sulfadoxine	+	3.44	310.9	311 > 156	15	311 > 92	25	0.42(8)	311 > 108	25	0.48(13)
Sulfamethorxazole	+	3.26	253.8	254 > 92	25	254 > 156	15	1.27(7)	254 > 108	20	0.56(6)
Trimethoprim	+	2.88	291.0	291 > 123	25	291 > 230	20	1.11(7)	291 > 261	25	0.83(5)
Valsartan	+	6.27	435.8	436 > 207	25	436 > 235	15	1.12(6)	436 > 261	15	<0.01(17)
Venlafaxine	+	4.61	278.1	278 > 58	15	278 > 260	10	0.43(5)	278 > 121	25	0.24(2)

Table 1 (Cont). MS/MS optimized conditions for selected compounds.

Compound	ESI	TR (min)	Precursor ion (m/z)	Q transition	C.E. (eV)	q1 transition	C.E. (eV)	q1/Q (RSD)	q2 transition	C.E. (eV)	q2/Q (RSD)
ILIS											
Acetaminophen-d_4	+	1.89	155.9	156 > 114	15						
Amphetamine-d_6	+	2.79	141.7	142 > 93	15						
Atorvastatin-d_5	+	6.67	563.9	564 > 445	20						
Benzoylecgonine-d_3	+	3.32	293.1	293 > 171	20						
Carbamazepine 10,11-epoxide-d_{10}	+	4.47	263.0	263 > 190	25						
Cocaethylene-d_8	+	4.23	326.0	326 > 204	20						
Cocaine-d_3	+	3.74	306.9	307 > 185	20						
Diclofenac-d_4	-	6.85	299.9	300 > 256	10						
MDMA-d_5	+	2.90	199.0	199 > 1650	10						
Omeprazole-d_3	+	5.22	348.8	349 > 198	10						
Salicylic acid-d_4	-	4.26	140.7	141 > 97	15						
Sulfamethoxazole-$^{13}C_6$	+	3.27	260.0	260 >162	15						
Trimethoprim-$13C_3$	+	2.87	294.1	294 >264	18						
Valsartan-d_8	+	6.24	443.9	444 > 207	15						

ES, electrospray ionization; TR, retention time; Q quantification; q confirmation, C.E. collision energy.

3.2. UHPLC conditions

In this work, different mobile phases (acetonitrile and methanol) with different composition (HCOOH and NH_4Ac at various concentrations) were tested. The effects of pH and ionic strength of the mobile phase on the peak shape, resolution and efficiencies were evaluated by varying the buffer concentration. Finally, a gradient consisting of water (solvent A) and MeOH (solvent B) both with 1 mM ammonium acetate and 0.01% formic acid was chosen as an appropriate mobile phase.

Initially, 10 µL were injected in the system as reference conditions. In order to further improve sensitivity, injection of increasing sample volumes was performed. On the basis of the column dimensions and the particle size (in this case, 2.1×100 mm, 1.7 µm), the dead volume of the column was estimated to be 400 µL. The recommended injection volume should not exceed the 10% of this dead volume, this is, 40 µL. Trying to perform LVI for this system, 50 and 100 µL were tested, obtaining satisfactory chromatographic peak shape in all cases. The best sensitivity was achieved when injecting 100 µL. Hence, the injection of 100 µL was selected for further validation.

3.3. Matrix effects: quantification

The high complexity and variability of the matrices in water samples (especially in wastewater samples) affected considerably the recovery values of some compounds. For almost half of the studied compounds, matrix effects resulting in ionization suppression were observed, being more important in EWW samples than in SW. Thus, acetaminophen and atorvastatin showed recoveries between 60 and 120% in the five SW tested, but decreased down to 27–60% in EWW. A few compounds experimented ionization enhancement due to co-eluted matrix components, leading to recoveries >100%. This was the case of levamisol, MDMA or trimethoprim. Among the different approaches proposed in the literature to remove or compensate for the matrix effects, the use of isotopically-labeled internal standards (if available) was considered the preferred option. Fourteen compounds could be corrected with their own ILIS, as they were available to our laboratory, obtaining satisfactory figures after correction, as expected. Erythromycin, levamisol, pravastatin, sulfadiazine and venlafaxine were corrected using an analog ILIS

(Table 2). The selection of analog ILIS was mainly based on chemical structure and/or retention time similarity between analyte and ILIS, as it was expected that both were affected by similar constituents of the matrix. In particular cases, e.g. erythromycin, an ILIS eluting at different retention time and with different chemical structure (sulfamethoxazole-$^{13}C_6$) was able to perform an efficient matrix effects correction, as previously reported by Gracia-Lor et al. [8]. The rest of the analytes were quantified using absolute response as matrix effects in the ten water samples tested were not much relevant.

Table 2. Results of the method validation for effluent wastewater (EWW) and surface water (SW). Limit of quantification (LOQ), recovery (%) and relative standard deviation at the three validation levels studied.

Compound	SW (n=5)				EWW (n=5)				ILIS used for correction
	Recovery (RSD) (both in %)			LOQ (ng L-1)	Recovery (RSD) (both in %)			LOQ (ng L-1)	
	10 ng L-1	100 ng L-1	1000 ng L-1		10 ng L-1	100 ng L-1	1000 ng L-1		
4-Acetyl aminoantipyrine	59 (16)[a]	79 (19)[a]	72 (5)[a]	0.8	95[b]	69[b]	78 (14)[a]	2.0	-
4-Aminoantipyrine	95 (12)[a]	74 (14)[a]	81 (9)	0.7	110[b]	66[b]	97[b]	0.4	-
4-Formyl aminoantipyrine	72 (13)[a]	105 (12)[a]	88 (4)[a]	1.9	120[b]	68[b]	82 (18)[a]	1.7	-
Acetaminophen	103 (17)[a]	111 (10)	107 (9)	1.1	131 (3)[a]	113 (15)	118 (7)	1.5	Acetaminophen-d_4
Alprazolam	88 (18)[a]	79 (10)	78 (8)	0.3	81 (16)[a]	74 (9)	77 (11)	1.2	-
Amphetamine	-	96 (12)	78 (19)	6.3	-	110 (11)	107 (11)	12.5	Amphetamine-d_6
Atorvastatin	84 (11)	85 (7)	100 (9)	0.8	92 (14)	92 (5)	109 (2)	0.8	Atorvastatin-d_5
Benzoylecgonine	88 (18)	83 (7)	97 (10)	0.1	88 (20)[a]	80 (16)	109 (2)	0.1	Benzoylecgonine-d_3
Bezafibrate	87 (20)[a]	83 (16)	95 (12)	1.3	82 (11)[a]	102 (24)[a]	111 (13)	2.1	-
Carbamazepine	81 (19)	65 (7)	91 (8)	0.2	77 (16)[a]	75 (15)	94 (8)	1.1	Carbamazepine 10,11-epoxide-d_{10}
Clarithromycin	93 (4)[a]	97 (14)[a]	90 (9)	2.9	117[b]	73 (17)[a]	81 (16)	4.1	-
Cocaethylene	89 (12)	93 (9)	97 (9)	0.7	100 (5)	93 (5)	102 (4)	0.8	Cocaethylene-d_8
Cocaine	77 (19)[a]	69 (11)	111 (10)	1.0	70 (9)	88 (17)	116 (3)	1.1	Cocaine-d_3
Diclofenac	-	82 (14)[a]	105 (5)	6.8	-	78 (17)[a]	104 (11)	7.2	Diclofenac-d_4
Enalapril	99 (17)	88 (6)	92 (5)	0.7	109 (9)	81 (4)	94 (8)	1.8	-
Erythromycin	115 (6)	85 (15)	72 (14)	0.8	125[b]	92 (23)[a]	94 (19)	2.1	Sulfamethoxazole-$^{13}C_6$
Florfenicol	69 (17)	91 (12)	83 (15)	2.2	97 (11)	84 (14)	109 (11)	8.6	-
Flumequine	87 (14)	87 (10)	113 (5)	0.4	90 (17)	73 (14)	97 (9)	1.2	-
Furaltadone	88 (11)	87 (10)	88 (10)	0.7	88 (16)	69 (16)	80 (17)	1.4	-
Gemfibrozil	83 (15)[a]	99 (12)[a]	92 (8)	2.3	103[b]	92[b]	90 (18)	1.8	-

[a] Validation performed for n=2-4, due to the high analyte concentration found in some "blank" samples.
[b] Recovery values without RSD mean (n=1).

Table 2 (Cont.). Results of the method validation for effluent wastewater (EWW) and surface water (SW). Limit of quantification (LOQ), recovery (%) and relative standard deviation at the three validation levels studied.

Compound	SW (n=5)				EWW (n=5)				ILIS used for correction
	Recovery (RSD) (both in %)			LOQ (ng L-1)	Recovery (RSD) (both in %)			LOQ (ng L-1)	
	10 ng L-1	100 ng L-1	1000 ng L-1		10 ng L-1	100 ng L-1	1000 ng L-1		
Irbesartan	86 (16)[a]	87 (13)[a]	97 (8)	0.2	115[b]	78 (12)[a]	99 (5)	1.0	-
Levamisol	83 (15)	95 (7)	101 (5)	0.2	87 (19)[a]	98 (16)	106 (10)	2.1	Cocaethylene-d_8
Lincomycin	84 (15)[a]	81 (17)[a]	104 (10)	0.1	88 (12)	78 (15)	75 (12)	0.4	-
Lorazepam	88 (14)[a]	82 (15)	86 (7)	3.1	109 (9)[a]	78 (20)[a]	94 (5)	4.5	-
MDMA	100 (10)	96 (6)	105 (8)	0.5	99 (18)	93 (2)	103 (8)	1.4	MDMA-d_5
Nalidixic acid	93 (14)	91 (9)	114 (6)	1.8	90 (17)	75 (13)	98 (9)	2.7	-
Naproxen	77 (18)[a]	70 (7)	80 (13)	11.7	62[b]	78[b]	85 (13)	7.3	-
Olanzapine	-	86 (1)[a]	108 (12)[a]	0.8	-	-	156 (13)	11.6	-
Omeprazole	103 (13)	89 (8)	98 (8)	0.2	118 (24)	95 (4)	102 (2)	1.1	Omeprazole-d_3
Oxolinic acid	96 (12)	83 (10)	86 (5)	1.8	98 (13)	70 (10)	80 (16)	2.9	-
Pantoprazole	93 (15)	99 (8)	103 (5)	0.1	93 (13)	81 (12)	105 (8)	0.8	-
Pravastatin	96 (14)	81 (13)	85 (13)	15.4	113[b]	82 (10)	83 (8)	16.7	Diclofenac-d_4
Roxithromycin	-	92 (4)[a]	83 (13)	5.6	-	95 (9)[a]	91 (16)	5.4	-
Salicylic acid	-	-	93 (15)	37.6	-	119[b]	84 (8)	41.1	Salicylic acid-d_4
Sulfadiazine	102 (16)	99 (9)	116 (8)	1.4	106 (19)	97 (7)	111 (12)	1.8	Sulfamethoxazole-$^{13}C_6$
Sulfadoxine	85 (12)	83 (11)	104 (7)	0.2	80 (18)	64 (12)	86 (16)	0.5	-
Sulfamethorxazole	96 (16)[a]	80 (10)	98 (11)	0.5	103[b]	83 (10)	106 (7)	0.8	Sulfamethoxazole-$^{13}C_6$
Trimethoprim	83 (14)	87 (13)	93 (10)	1.8	111[b]	81 (19)	104 (10)	2.3	Trimethoprim-$^{13}C_3$
Valsartan	74 (11)	88 (4)	98 (12)	3.8	114[b]	98 (18)[a]	89 (14)[a]	4.2	Valsartan-d_8
Venlafaxine	79 (21)[a]	78 (15)	102 (11)	0.2	111[b]	88 (19)[a]	100 (6)	1.0	Atorvastatin-d_5

[a]Validation performed for n=2-4, due to the high analyte concentration found in some "blank" samples.
[b] Recovery values without RSD mean (n=1).

3.4. Method validation

Analytical characteristics of the method were evaluated in two types of water samples: five surface water and five effluent wastewater samples, spiked at three concentration levels each (10, 100 and 1000 ng/L).

The linearity of the method was studied in the range 1–2500 ng/L for all compounds. Calibration curves showed in all cases correlation coefficients greater than 0.99, and residuals lower than 25%.

Accuracy and precision were estimated from injection of different water samples spiked at the three concentrations indicated above. All the "blank" samples contained at least one or more target analytes. Thus, the samples were previously analyzed and those with lower drug concentration were selected as "blank" samples for method validation. Concentration of target compounds found in these "blank" samples were subtracted from the spiked samples.

The results obtained for most compounds were satisfactory at the three validation levels, with recoveries between 70 and 120% and precision (RSD) below 20% (Table 2). At the lowest level (10 ng/L) amphetamine, diclofenac, olanzapine, roxithromycin and salicylic acid could not be validated, due to their lower sensitivity. For some compounds, validation was not feasible in all the samples tested due to the high analyte concentration found in different "blank" samples (e.g. the three dipyrone metabolites or gemfibrozil). In these cases, the number of data used in validation was less than 10 (5 SW and 5 EWW) (highlighted as [a] or [b] in Table 2).

The method presented satisfactory precision for most compounds with RSDs below 20% at the three fortification levels. Regarding LOQ, they were ≤3 ng/L for 32 out of 40 compounds in SW. For another 5 analytes LOQs ranged from 3 to 7 ng/L, and for the remaining 3 were slightly higher, between 12 and 38 ng/L. In EWW, 29 compounds presented LOQs ≤3 ng/L, 7 ranged from 3 to 9 ng/L and the remaining 4 were between 12

and 41 ng/L. According to our data, it seems that the type of water did not much affect the attainable sensitivity despite of being a direct injection method.

3.5. Analysis of water samples

To demonstrate the applicability of the method developed, 10 effluent wastewater and 10 surface water samples were analyzed. In every sequence of analysis, a calibration curve in solvent was injected at the beginning and at the end of the batch sample. Quality controls (QCs) were also included in every sequence, consisting on selected EWW and SW samples spiked with all pharmaceuticals at 100 ng/L. QC recoveries were satisfactory (in the range of 70–120%) for the majority of the compounds. However, QCs recoveries for venlafaxine (using atorvastatin-d_5 as IS) and for levamisol (using cocaethylene-d_8) were around 130%. As it has been already reported in the literature, the use of analogs IS does not always assure an efficient matrix effects correction [40] and [41].

Identification of positive findings was supported by evaluation of q_1/Q and q_2/Q ratios. The finding was considered as positive when retention time and at least one experimental ion-ratio were within the established tolerances [42], when compared with a reference standard. Although the acquisition of two SRM transitions per compound together with the accordance in the retention time are normally considered sufficient for a reliable confirmation of the compound identity, in this work three transitions were acquired in order to increase the confidence of the confirmation process [40]. Using three transitions, one can minimize the possibilities of reporting false negatives when the ion ratio is not accomplished, in those cases where one of the transitions seems to be interfered. As an example, Fig. 1 shows positive findings of alprazolam, bezafibrate and sulfamethoxazole in EWW. As it can be seen, the three transitions showed a peak at the same retention time. Moreover, at least one q/Q ratio was within tolerance limits.

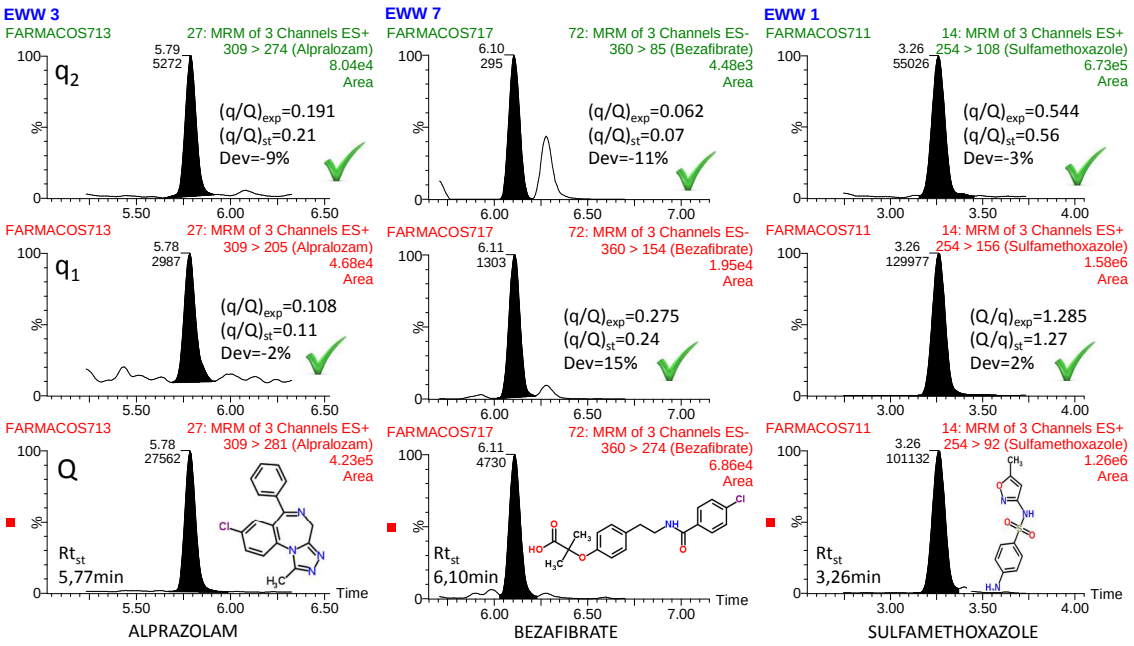


Figure 1. UHPLC-MS/MS chromatograms detected in EWW samples.

Table 3 and Table 4 show the concentration values (ng/L) found for each compound in EWW and SW, respectively. 32 analytes were detected in the 10 EWWs analyzed, illustrating the frequent occurrence of drugs in wastewater samples and the fact that many of them are not completely removed in WWTPs. Carbamazepine, used for the treatment of epilepsy and bipolar disorder, was the compound most frequently detected, appearing in all samples analyzed. This was followed by the angiotensin II antagonist valsartan and 4-acetyl aminoantipyrine (metabolite of the analgesic dipyrone), which were present in 90% of EWWs. 4-formyl aminoantipyrine (another metabolite of dypirone), the anthelmintic levamisol, the antibiotics sulfamethoxazole and trimethoprim, and the antidepressant venlafaxine appeared in 80% of EWWs. The highest concentrations corresponded to 4-acetyl aminoantipyrine (7.2 µg/L), valsartan (4.6 µg/L), 4-formyl aminoantipyrine (3.2 µg/L) and the analgesic naproxen (1.9 µg/L). In these cases, samples were diluted and re-analyzed to fit the linear range of the method.

Table 3. Summary of the results obtained for target pharmaceuticals in EWW, applying the analytical methodology described in this article. Between brackets, the concentrations obtained using the on-line SPE-LC-MS/MS alternative method.

Compound	EWW (ng/L)									
	1	2	3	4	5	6	7	8	9	10
4-Aminoantipyrine*	9	15	<LOQ	-	<LOQ	40	43	14	-	26
Acetaminophen*	-	-	-	14	-	45	<LOQ	-	8	-
Amphetamine*	-	21	-	-	29	-	-	-	-	-
Benzoylecgonine*	<LOQ	40 (48)	6	<LOQ	656 (735)	11	100 (127)	43 (54)	<LOQ	43 (50)
Clarithromycin*	-	14	26	-	34	<LOQ	27	15	-	-
Cocaethylene*	-	<LOQ	-	-	15	<LOQ	8	-	-	<LOQ
Cocaine*	9	<LOQ	-	<LOQ	72 (54)	12	-	12	8	24
Diclofenac*	-	266 (251)	884 (1115)	-	216 (313)	158 (241)	845 (1181)	300 (386)	-	212 (322)
Erythromycin*	13	55	-	-	37	18	49	14	-	25
Flumequine*	-	-	<LOQ	-	-	-	-	-	-	7
MDMA*	-	45	-	-	45	<LOQ	-	22	-	48
Nalidixic acid*	-	-	17	-	<LOQ	-	-	-	-	8
Naproxen*	-	42	32	-	1942 (3007)	-	515 (642)	<LOQ	-	357 (419)
Oxolinic acid*	-	-	-	-	-	-	-	-	-	5
Pantoprazole*	-	5	2	-	<LOQ	4	4	7	-	4
Sulfadiazine*	-	-	28	-	-	-	-	10	-	-
Sulfamethoxazole*	89	35	372 (308)	<LOQ	19	21	29	25	-	29
Trimethoprim*	15	83	9	-	75	4	13	86	-	25
Venlafaxine*	414 (366)	316 (282)	421 (389)	-	343 (457)	263 (314)	252 (265)	201 (208)	<LOQ	239 (260)
4-Acetyl aminoantipyrine	77	3032	253	-	7239	197	1357	2298	18	689
4-Formyl aminoantipyrine	860	1583	3425	-	3208	766	1898	1235	<LOQ	853
Alprazolam	14	11	17	-	<LOQ	12	13	10	-	12
Atorvastatin	-	7	-	-	16	-	-	-	-	<LOQ
Bezafibrate	-	29	-	-	87	10	35	16	-	53
Carbamazepine	112	52	119	3	135	64	149	54	2	90
Gemfibrozil	-	765	4	-	538	25	507	365	<LOQ	95
Irbesartan	-	531	<LOQ	-	506	404	799	266	<LOQ	484
Levamisol	44	311	155	-	150	163	768	178	-	497
Lincomycin	-	-	-	-	<LOQ	-	6	109	-	7
Lorazepam	-	52	-	-	109	58	81	46	-	74
Pravastatin	-	16	-	-	-	-	<LOQ	<LOQ	-	-
Valsartan	41	2864	54	-	4575	291	1457	246	13	399

*Compounds also analyzed by the on-line SPE LC-MS/MS methodology described in section -: not detected

Table 4. Summary of the results obtained for target pharmaceuticals in SW, applying the analytical methodology described in this article. Between brackets, the concentrations obtained using the on-line SPE-LC-MS/MS alternative method.

Compound	SW (ng/L)									
	1	2	3	4	5	6	7	8	9	10
Acetaminophen*	-	-	-	480 (654)	9	13	13	12	32 (10)	10
Benzoylecgonine*	<LOQ	<LOQ	18 (8)	31 (23)	6	<LOQ	<LOQ	7	6	6
Clarithromycin*	-	-	11	34 (45)	-	-	-	-	-	-
Cocaethylene*	-	-	-	7	-	-	-	-	-	-
Cocaine*	8	<LOQ	8	14 (8)	-	8	8	10 (5)	-	<LOQ
Diclofenac*	-	34 (24)	135 (99)	14	-	-	-	-	-	-
Erythromycin*	-	-	10	<LOQ	-	-	-	-	-	-
Flumequine*	3	-	-	-	-	-	-	<LOQ	-	-
Levamisol*	-	4	76 (44)	5	-	-	-	-	-	-
MDMA*	-	-	15	13	-	-	-	-	-	-
Nalidixic acid*	3	-	-	<LOQ	-	-	-	4	-	-
Naproxen*	-	-	67 (56)	114 (172)	-	-	-	-	-	-
Oxolinic acid*	5	-	-	-	-	-	-	-	-	<LOQ
Pantoprazole*	-	1	-	-	-	-	-	-	-	-
Sulfamethoxazole*	-	21	25	11	13	<LOQ	-	-	-	<LOQ
Trimethoprim*	3	-	<LOQ	5	-	-	-	-	-	-
Venlafaxine*	9	244 (217)	93 (61)	30 (18)	16	10	<LOQ	<LOQ	9	16
4-Acetyl aminoantipyrine	-	<LOQ	719	182	8	9	6	<LOQ	-	21
4-Formyl aminoantipyrine	-	9	663	101	33	13	-	<LOQ	-	60
Alprazolam	-	11	8	<LOQ	-	-	-	-	-	-
Carbamazepine	<LOQ	73	22	10	7	-	2	2	-	6
Gemfibrozil	-	-	105	80	-	-	-	-	-	-
Irbesartan	-	<LOQ	5	40	-	-	-	-	-	6
Lincomycin	-	12	5	1	-	-	-	-	-	-
Lorazepam	-	13	18	<LOQ	-	-	-	-	-	44
Valsartan	-	-	13	224	-	-	-	-	-	-

*Compounds also analyzed by the on-line SPE LC-MS/MS methodology described in section -: not detected

In relation to surface water samples, up to 26 compounds were detected in the samples analyzed. All these compounds were also found in EWWs, normally at higher concentrations. Venlafaxine and acetaminophen were the compounds most frequently detected, being present in 80% and 70% of the samples, respectively. 4-acetyl, 4-formyl aminoantipyrine, cocaine and its metabolite benzoylecgonine, were present in 60% of SWs analyzed. The highest concentration corresponded to dipyrone metabolites 4-formyl (0.72 µg/L) and 4-acetyl aminoantipyrine (0.66 µg/L).

As an illustrative example, Fig. 2 shows UHPLC–MS/MS chromatograms for SW 4 (only the quantitative transition Q is shown), which was positive for 19 out of the 40 target compounds. Concentration data for this sample are shown in Table 4, where it can be seen that acetaminophen presented the highest value (480 ng/L). Four drugs of abuse (benzoylecgonine, cocaethylene, cocaine and MDMA) were also detected in the range of 7–31 ng/L. These figures reveal that licit and illicit drugs can actually reach surface water due to the incomplete removal in WWTPs.

3.5.1. On-line SPE HPLC-ESI-MS/MS

The same 20 samples were analyzed by another laboratory that applied an analytical methodology based on on-line SPE-LC coupled to triple quadrupole mass spectrometry. With this methodology, only 25 human and veterinary pharmaceuticals and drugs of abuse were included in the target method. All of them were determined with ESI operating in positive ionization mode. For confirmation, two SRM transitions at the same retention time, and the accomplishment of the q/Q ratios were required. Regarding quantification parameters, two internal standards were used to correct possible deviations: diclofenac-$^{13}C_6$ for pharmaceuticals and cocaine-d_3 for drugs of abuse. The linearity of the method was studied in the range 2–150 ng/L for all compounds. The method presented satisfactory accuracy and precision for all compounds, with recoveries values >85% and RSDs below 13%. Regarding LOQ, they ranged from 2 to 20 ng/L for SW and from 40 to 400 ng/L for EWW. The on-line SPE-LC method was implemented in this laboratory under requirements of ISO-170025 [34].

Data obtained are also shown in Table 3 and Table 4 (between brackets). Six compounds (cocaine, benzoylecgonine, diclofenac, naproxen, sulfamethoxazole and venlafaxine) were found in EWW samples, less than in the direct injection methodology (32 compounds). This was surely due to the higher LOQs obtained in the on-line procedure, due to the dilution step (1/20) applied to EWW samples prior to on-line SPE. The concentration values ranged from 0.048 to 3.1 µg/L and were in agreement with the results obtained by the direct injection approach. Regarding surface water samples, where no dilution was performed, up to 8 compounds could be detected (benzoylecgonine, cocaine, acetaminophen, clarithromycin, diclofenac, levamisol, naproxen and venlafaxine). Among them, venlafaxine was the compound most frequently detected (3 out of 10 SW analyzed), and the highest concentration found was for acetaminophen (0.65 µg/L). All concentration values obtained by this methodology were also in accordance with the results reported after direct injection analyses.

Except for the differences due to the distinct sensitivity of the two procedures, the concentrations found by both of them for the wide majority of positive samples were rather similar, supporting the applicability and reliability of our more-sensitive large-volume direct injection approach.

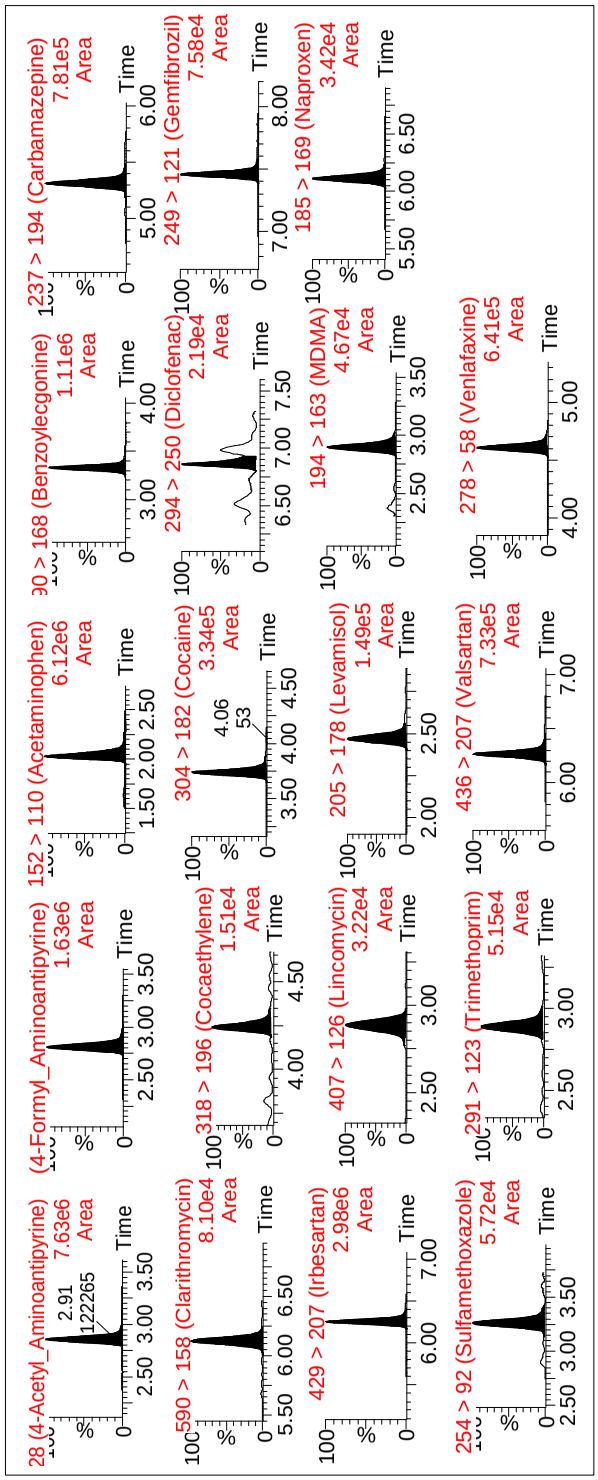


Figure 2. UHPLC-MS/MS chromatograms (Q transition) for a surface water sample (*SW4*, see Table 4) where 19 target compounds were found. Positive/negative voltage switching mode applied within the same run.

4. CONCLUSIONS

Analytical methodology based on UHPLC–MS/MS QqQ has been developed for the simultaneous quantification and confirmation of 40 human and veterinary pharmaceuticals and drugs of abuse in effluent wastewater and surface samples. The direct injection of water samples (100 µL), without any previous sample treatment, has been shown as an attractive approach as it avoids time-consuming sample preparation steps and reduces the amounts of solvents used. The determination of target compounds was performed in positive/negative voltage switching mode in a single chromatographic run of only 10 min. With a few exceptions, a highly reliable identification of the compounds was feasible thanks to the acquisition of three SRM transitions per compound and the accomplishment of the ion ratio and retention time deviations. Satisfactory accuracy and precision were obtained in recovery experiments at three concentration levels in two kinds of water matrices, EWW and SW, using 10 different samples to this aim. The LOQs were in most cases lower than 3 ng/L. The application of this method to 10 effluent wastewater and 10 surface samples, allowed the detection of 32 and 26 compounds, respectively. Carbamazepine was the compound most frequently detect (100%) in EWW and venlafaxine (80%) in SW samples. This methodology has been proven to be an attractive and efficient approach for rapid determination of pharmaceuticals and drugs of abuse in environmental water, achieving low LOQs without the need for a preliminary pre-concentration step.

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REFERENCES

[1] D. Fatta-Kassinos, S. Meric, A. Nikolaou, Pharmaceutical residues in environmental waters and wastewater: Current state of knowledge and future research, Anal. Bioanal. Chem. 399 (2011) 251–275.

[2] I. Senta, S. Terzic, M. Ahel, Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment, Water Res. 47 (2013) 705–714.

[3] S. Castiglioni, R. Bagnati, M. Melis, D. Panawennage, P. Chiarelli, R. Fanelli, et al., Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine, Water Res. 45 (2011) 5141–5150.

[4] S. Ortiz de García, G. Pinto Pinto, P. García Encina, R. Irusta Mata, Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain, Sci. Total Environ. 444 (2013) 451–465.

[5] N. Dorival-García, A. Zafra-Gómez, S. Cantarero, A. Navalón, J.L. Vílchez, Simultaneous determination of 13 quinolone antibiotic derivatives in wastewater samples using solid-phase extraction and ultra performance liquid chromatography–tandem mass spectrometry, Microchem. J. 106 (2013) 323–333.

[6] D.R. Baker, B. Kasprzyk-Hordern, Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: new developments., Sci. Total Environ. 454-455 (2013) 442–56.

[7] R. López-Serna, A. Jurado, E. Vázquez-Suñé, J. Carrera, M. Petrović, D. Barceló, Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain., Environ. Pollut. 174 (2013) 305–15.

[8] E. Gracia-Lor, J.V. Sancho, F. Hernández, Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A. 1218 (2011) 2264–2275.

[9] R. López-Serna, M. Petrovic, D. Barceló, Development of a fast instrumental method for the analysis of pharmaceuticals in environmental and wastewaters based on ultra high performance liquid chromatography (UHPLC)-tandem mass spectrometry (MS/MS), Chemosphere. 85 (2011) 1390–1399.

[10] S. Bayen, X. Yi, E. Segovia, Z. Zhou, B.C. Kelly, Analysis of selected antibiotics in surface freshwater and seawater using direct injection in liquid chromatography

electrospray ionization tandem mass spectrometry., J. Chromatogr. A. 1338 (2014) 38–43.

[11] L. Bijlsma, J.V. Sancho, E. Pitarch, M. Ibáñez, F. Hernández, Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater, J. Chromatogr. A. 1216 (2009) 3078–3089.

[12] S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati, Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry, Anal. Chem. 78 (2006) 8421–8429.

[13] S. Huntscha, H.P. Singer, C.S. McArdell, C.E. Frank, J. Hollender, Multiresidue analysis of 88 polar organic micropollutants in ground, surface and wastewater using online mixed-bed multilayer solid-phase extraction coupled to high performance liquid chromatography-tandem mass spectrometry., J. Chromatogr. A. 1268 (2012) 74–83.

[14] A.C. Chiaia, C. Banta-Green, J. Field, Eliminating solid phase extraction with large-volume injection LC/MS/MS: Analysis of illicit and legal drugs and human urine indicators in US wastewaters, Environ. Sci. Technol. 42 (2008) 8841–8848.

[15] F. Busetti, W.J. Backe, N. Bendixen, U. Maier, B. Place, W. Giger, et al., Trace analysis of environmental matrices by large-volume injection and liquid chromatography-mass spectrometry, Anal. Bioanal. Chem. (2011) 1–12.

[16] J.-D. Berset, R. Brenneisen, C. Mathieu, Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography - Electrospray tandem mass spectrometry (HPLC-MS/MS), Chemosphere. 81 (2010) 859–866.

[17] M.M. Galera, P.P. Vázquez, M.D.M.P. Vázquez, M.D.G. García, C.F. Amate, Analysis of ß-blockers in groundwater using large-volume injection coupled-column reversed-phase liquid chromatography with fluorescence detection and liquid chromatography time-of-flight mass spectrometry, J. Sep. Sci. 34 (2011) 1796–1804.

[18] M.J. Martínez Bueno, S. Uclés, M.D. Hernando, A.R. Fernández-Alba, Development of a solvent-free method for the simultaneous identification/quantification of drugs of abuse and their metabolites in environmental water by LC-MS/MS, Talanta. 85 (2011) 157–166.

[19] E. Zuccato, S. Castiglioni, R. Fanelli, Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment, J. Hazard. Mater. 122 (2005) 205–209.

[20] A.L.N. Van Nuijs, I. Tarcomnicu, W. Simons, L. Bervoets, R. Blust, P.G. Jorens, et al., Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater, Anal. Bioanal. Chem. 398 (2010) 2211–2222.

[21] S. González Alonso, M. Catalá, R.R. Maroto, J.L.R. Gil, Á.G. de Miguel, Y. Valcárcel, Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain), Environ. Int. 36 (2010) 195–201.

[22] M.D. Hernando, M.J. Gómez, A. Agüera, A.R. Fernández-Alba, LC-MS analysis of basic pharmaceuticals (beta-blockers and anti-ulcer agents) in wastewater and surface water, Pharm. Anal. 26 (2007) 581–594.

[23] R. Rosal, A. Rodríguez, J.A. Perdigón-Melón, A. Petre, E. García-Calvo, M.J. Gómez, et al., Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation, Emerg. Contam. Water Occur. Fate, Remov. Assess. Water Cycle (from Wastewater to Drink. Water). 44 (2010) 578–588.

[24] M.A. Sousa, C. Gonçalves, E. Cunha, J. Hajšlová, M.F. Alpendurada, Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS, Anal. Bioanal. Chem. 399 (2011) 807–822.

[25] Y. Valcárcel, S. González Alonso, J.L. Rodríguez-Gil, A. Gil, M. Catalá, Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk, Chemosphere. 84 (2011) 1336–1348.

[26] M. Gros, M. Petrovic, A. Ginebreda, D. Barceló, Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes, Environ. Int. 36 (2010) 15–26.

[27] M. Gros, S. Rodríguez-Mozaz, D. Barceló, Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem, J. Chromatogr. A. 1248 (2012) 104–21.

[28] R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández, Qualitative validation of a liquid chromatography-quadrupole-time of flight mass spectrometry screening method for organic pollutants in waters, J. Chromatogr. A. 1276 (2013) 47–57.

[29] M. José Gómez, O. Malato, I. Ferrer, A. Agüera, A.R. Fernández-Alba, Solid-phase extraction followed by liquid chromatography-time-of-flight- mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study, J. Environ. Monit. 9 (2007) 719–729.

[30] F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez, Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry, Anal. Chim. Acta. 684 (2011) 87–97.

[31] L. Bijlsma, E. Emke, F. Hernández, P. de Voogt, Performance of the linear ion trap Orbitrap mass analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water, Anal. Chim. Acta. 768 (2013) 102-110.

[32] C.L. Chitescu, E. Oosterink, J. de Jong, A.A.M. Linda Stolker, Accurate mass screening of pharmaceuticals and fungicides in water by U-HPLC-Exactive Orbitrap MS., Anal. Bioanal. Chem. 403 (2012) 2997–3011.

[33] F. Wode, C. Reilich, P. van Baar, U. Dünnbier, M. Jekel, T. Reemtsma, Multiresidue analytical method for the simultaneous determination of 72 micropollutants in aqueous samples with ultra high performance liquid chromatography-high resolution mass spectrometry., J. Chromatogr. A. 1270 (2012) 118–26.

[34] IPROMA SOP. Determinación de fármacos y drogas en agua de consumo, continental y residual empleando SPE-on-line y HPLC-MS/MS.

[35]http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf (Accessed on March 2011).

[36] M. Farré, M. Petrovic, D. Barceló, Recently developed GC/MS and LC/MS methods for determining NSAIDs in water samples., Anal. Bioanal. Chem. 387 (2007) 1203–14.

[37] L. Penney, C. Bergeron, B. Coates, A. Wijewickreme, Simultaneous determination of residues of dipyrone and its major metabolites in milk, bovine muscle, and porcine muscle by liquid chromatography/mass spectrometry, J. AOAC Int. 88 (2005) 496–504.

[38] H. Ergün, D.A.C. Frattarelli, J. V Aranda, Characterization of the role of physicochemical factors on the hydrolysis of dipyrone., J. Pharm. Biomed. Anal. 35 (2004) 479–87.

[39] M. Ibáñez, E. Gracia-Lor, J.V. Sancho, F. Hernández, 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, J Mass Spectr, 47 (2012) 1040-1046.

[40] T. Benijts, R. Dams, W. Lambert, A. De Leenheer, Countering matrix effects in environmental liquid chromatography- electrospray ionization tandem mass spectrometry water analysis for endocrine disrupting chemicals, J. Chromatogr. A. 1029 (2004) 153-159.

[41] E. Gracia-Lor, J.V. Sancho, F. Hernández, Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry, J. Chromatogr. A. 1217 (2010) 622–632.

[42] (2002) European Union Decision 2002/657/EC Off. J. Eur. Commun., L221 pp. 8-36 (12 August 2002).

2.3 Discusión de los resultados

2.3.1 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. Para ello, se inyectaron los patrones de referencia con el objetivo de obtener el espectro en modo *full scan* y en modo MS/MS a diferentes energías de cono y de colisión. A modo de ejemplo, en la **Figura 2.1** se observan los espectros MS/MS obtenidos a diferentes energías de colisión (10, 15, 20 y 25 eV) para el MDMA y el bezafibrato. La mayor parte de los compuestos se determinaron en modo de ionización positivo (33 de 40 compuestos), y tan solo 7 mostraban mejor sensibilidad en modo negativo. En todos los casos, se seleccionó como ion precursor el $[M+H]^+$ o $[M-H]^-$, excepto para el naproxeno. Este compuesto se fragmenta fácilmente en la fuente de ionización, dando los iones 169 y 185. Este último, debido a su mayor abundancia, se seleccionó como ion precursor para la transición de cuantificación (Q: 185 →169).

Se optimizaron 3 transiciones por compuesto, con el fin de aumentar la confianza del proceso de identificación/confirmación. Para 3 analitos, ácido salicílico, gemfibrozil y naproxeno, solamente fue posible seleccionar 1 o 2 transiciones, debido a la escasa presencia de iones producto. En la medida de lo posible, se evitaron las transiciones poco selectivas (pérdidas de agua), con el objetivo de aumentar la selectividad del método y disminuir la posibilidad de que existan interferencias que podrían dar lugar a falsos positivos, o, con más probabilidades, falsos negativos en el caso de que sólo una de las transiciones estuviera interferida, con lo que no se cumpliría la relación iónica (*ion ratio*) esperada.

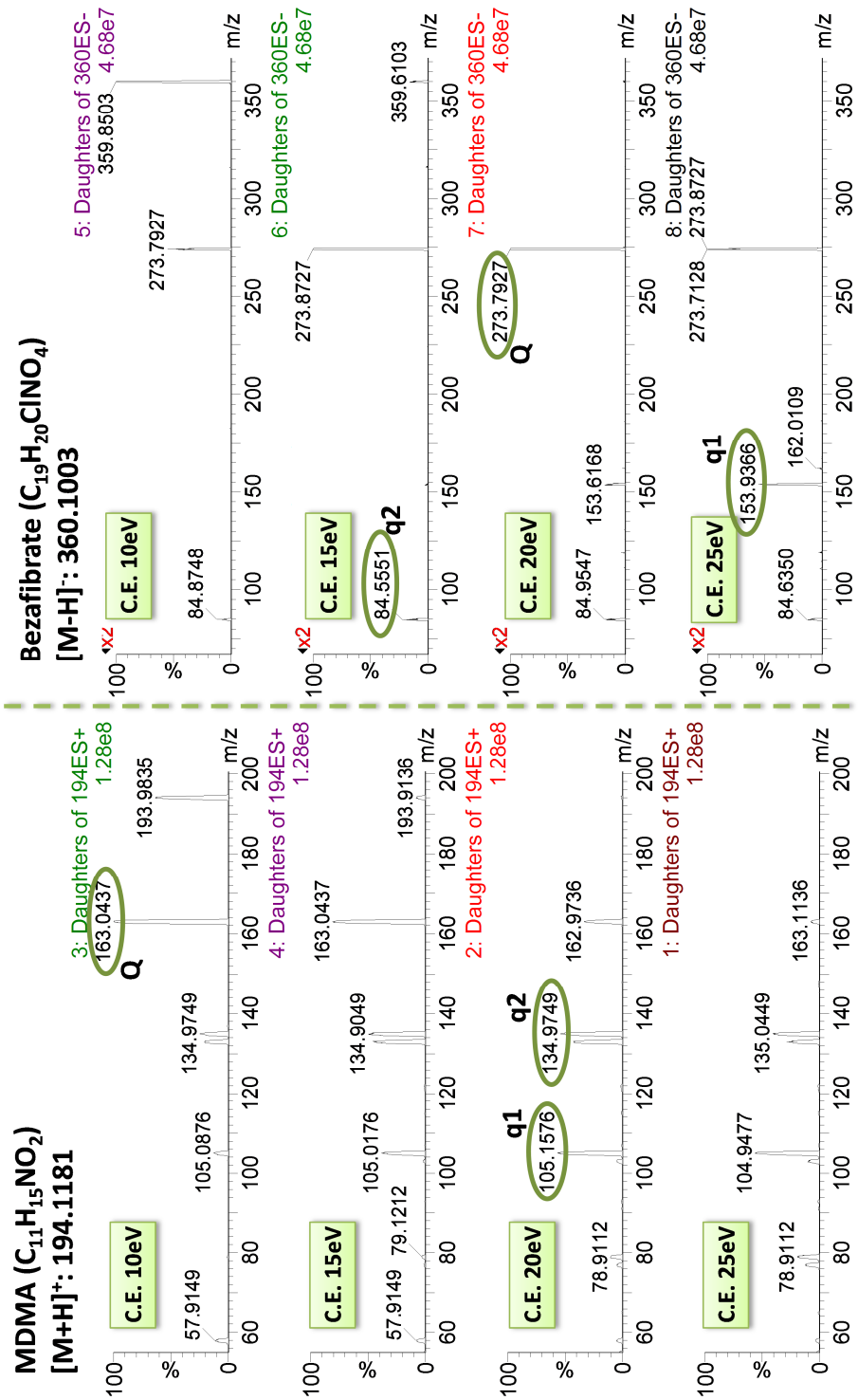

Figura 2.1 Optimización de la energía de colisión para el MDMA y el bezafibrato.

2.3.2 Optimización cromatográfica

Para seleccionar la fase móvil se probaron varios disolventes orgánicos (metanol y acetonitrilo) con distintos aditivos (ácido fórmico (HCOOH) y acetato amónico (NH_4Ac)) a varias concentraciones. Con el propósito de analizar simultáneamente todos los analitos, es decir, tanto los que se ionizan en modo 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, NH_4Ac (1 mM) mejoraba la sensibilidad de los compuestos medidos en modo positivo, mientras que HCOOH (0.01%) mejoraba la separación cromatográfica de estos y, al mismo tiempo, favorecía la retención en la columna de los compuestos ionizados en modo negativo.

Otro parámetro que ayudó a mejorar la sensibilidad fue el volumen de inyección. Se probaron 10, 50 y 100 μL y tras observar una adecuada forma de pico para todos los volúmenes, se eligió inyectar 100 μL, pues se conseguía mayor sensibilidad. La **Figura 2.2** muestra tres ejemplos (cocaetileno, pravastatina y pantoprazol) que ilustran cómo la inyección de 100 μL proporciona mayor sensibilidad que volúmenes menores de inyección.

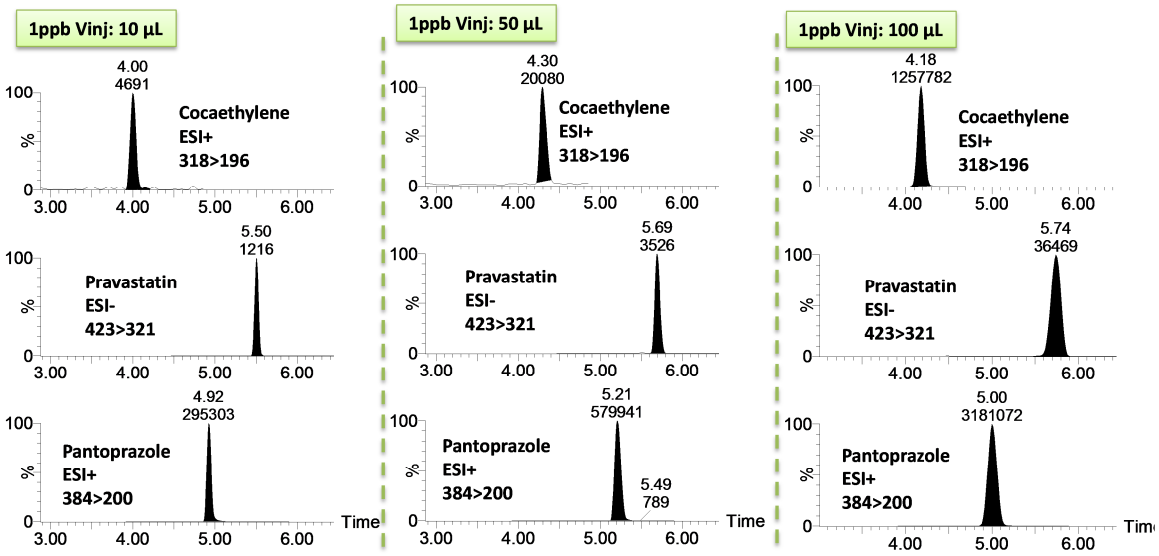

Figura 2.2 Optimización del volumen de inyección.

2.3.3 Validación del método

Tras optimizar las variables experimentales, se llevó a cabo la validación del método. Para ello se evaluaron el rango de linealidad, la exactitud, la precisión y el límite de cuantificación (*Limit Of Quantification*, LOQ) en cada una de las matrices estudiadas.

El estudio de la linealidad se realizó mediante la inyección por triplicado de ocho disoluciones patrón, en el rango comprendido entre 1 y 2500 ng/L, presentando en todos los casos coeficientes de correlación (r^2) mayores de 0.99 y residuales inferiores a ±25%.

La exactitud del método se estimó mediante ensayos de recuperación en diez aguas distintas (5 superficiales y 5 efluentes). Dichas muestras se fortificaron a 3 niveles de concentración 10, 100 y 1000 ng/L, cada una. La *Tabla 2* del *Artículo Científico 1* muestra valores satisfactorios de exactitud (recuperaciones entre el 70-120%) y

precisión (desviaciones estándares relativas menores del 20%) para la gran mayoría de compuestos estudiados.

A modo de ejemplo, la **Figura 2.3** muestra la correcta detección del bezafibrato, carbamazepina, cocaína y sulfadoxina, utilizando 3 transiciones, en agua superficial al nivel más bajo de validación (10 ng/L).

Cabe comentar que 5 compuestos, anfetamina, diclofenaco, olanzapina, roxitromicina y ácido salicílico, no pudieron ser validados al nivel más bajo (10 ng/L), debido a la poca sensibilidad para los mismos (el ácido salicílico tampoco al nivel intermedio, 100 ng/L). La presencia de ciertos compuestos seleccionados en las muestras "blanco" empleadas en la validación, supuso una dificultad adicional 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 la mayoría de compuestos los LOQ calculados fueron inferiores a 3 ng/L.

Para contrarrestar el efecto matriz observado en las muestras, se utilizaron varios analitos marcados isotópicamente. En general, la corrección se realizó usando el propio analito marcado isotópicamente, cuando éste estaba disponible. Para aquellos compuestos donde la corrección era necesaria y no se disponía de su patrón marcado isotópicamente (IS) (eritromicina, levamisol, pravastatina, sulfadiazina y venlafaxina), la elección de un patrón interno análogo se hizo en función de la estructura química y/o del tiempo de retención. Como puede observarse en la *Tabla 2, Artículo Científico 1*, el uso de IS condujo a una corrección satisfactoria en la mayoría de los analitos.

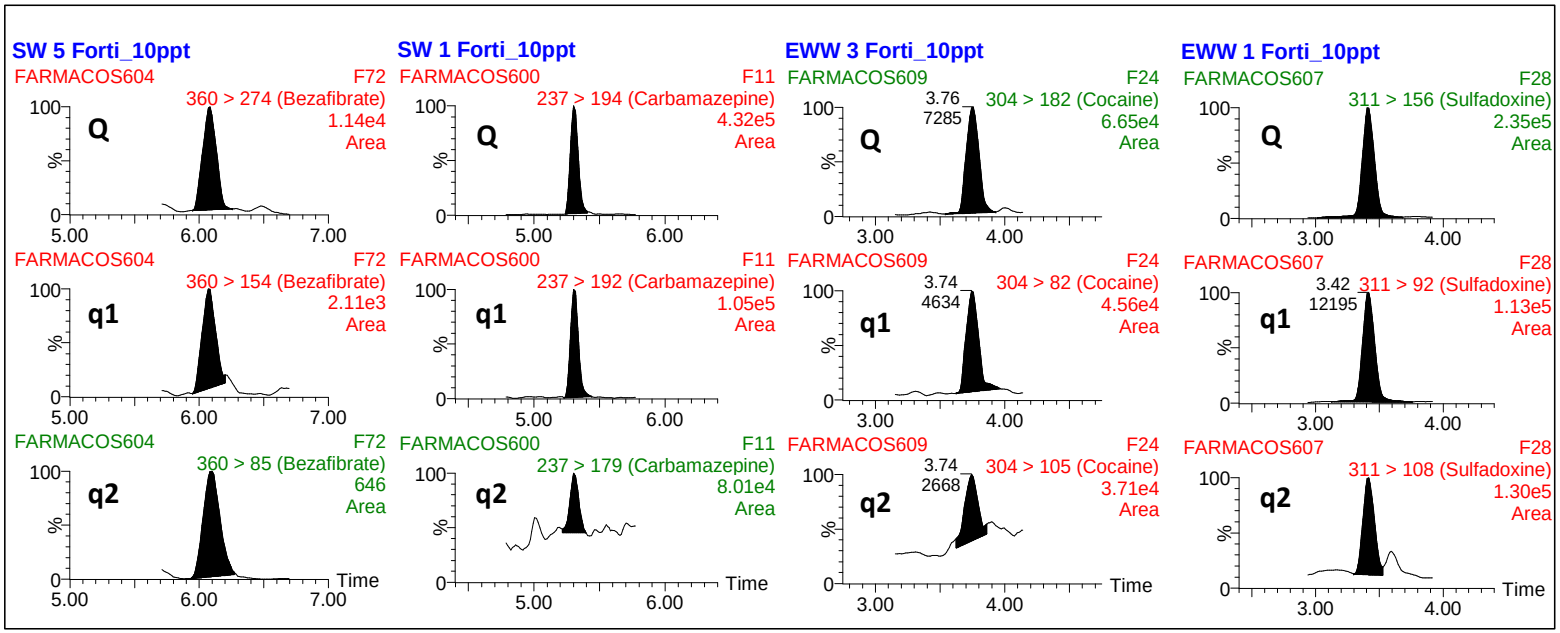

Figura 2.3 Cromatogramas UHPLC-MS/MS del bezafibrato, carbamazepina, cocaína y sulfadoxina en una agua superficial al nivel de validación de 10 ng/L.

2.3.4 Análisis de muestras reales

La metodología desarrollada se aplicó a 20 muestras de agua, 10 residuales de efluente y 10 superficiales, y permitió la detección de 32 y 26 compuestos, respectivamente. Entre los más frecuentemente detectados, y a mayores concentraciones, se encontrarían los metabolitos de la dipirona: 4-acetil aminoantipirina (max concentración 7.20 μg/L), 4-formil aminoantipirina (max concentración 3.42 μg/L) y 4-aminoantipirina (max concentración 0.04 μg/L).

Por lo que se refiere a estos metabolitos, todos ellos comparten iones producto y podrían co-eluir sin una adecuada separación cromatográfica, pudiendo llegar a provocar una errónea cuantificación. Tal y como reportó Ibáñez et al. (2012), 4-AAA y 4-FAA sufren una fragmentación en la fuente generando el ion *m/z* 204, que corresponde precisamente al $[M+H^+]$ del 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 ello, es importante llevar a cabo una adecuada optimización del gradiente cromatográfico, así como una correcta selección de las transiciones SRM. La **Figura 2.4** muestra cómo los tres metabolitos indicados se pudieron separar de modo satisfactorio en este trabajo. Cabe destacar que la adquisición simultánea de tres transiciones por compuesto resultó muy útil a la hora de confirmar sus identidades.

Tal y como se muestra en las *Tablas 3* y *4*, *Artículo Científico 1*, valsartán y 4-acetil aminoantipirina fueron detectados en 9 de las 10 aguas de efluente analizadas. Por otro lado, la venlafaxina fue el fármaco más veces detectado en aguas superficiales. Cabe destacar que 4-aminoantipirina, anfetamina, sulfadiazina, atorvastatina, bezafibrato y pravastatina se detectaron en las muestras de efluente, pero no en aguas superficiales.

De los 40 compuestos estudiados en este trabajo, 8 fármacos (enalapril, florfenicol, furaltadona, olanzapina, omeprazol, roxitromicina, ácido salicílico y sulfadoxina) no se detectaron en ninguna de las muestras de agua analizadas. Entre ellos, se encuentra el omeprazol, cuya presencia en el medio ambiente cabría esperar, ya que es uno de los fármacos más consumidos según datos oficiales proporcionados por el Ministerio de Sanidad Español. Tras estos resultados surgió un nuevo trabajo sobre el comportamiento del omeprazol en el medio ambiente, donde se llevaron a cabo experimentos tanto de degradación como de metabolismo, con el fin de identificar los biomarcadores más adecuados para detectar su presencia en el medio ambiente.

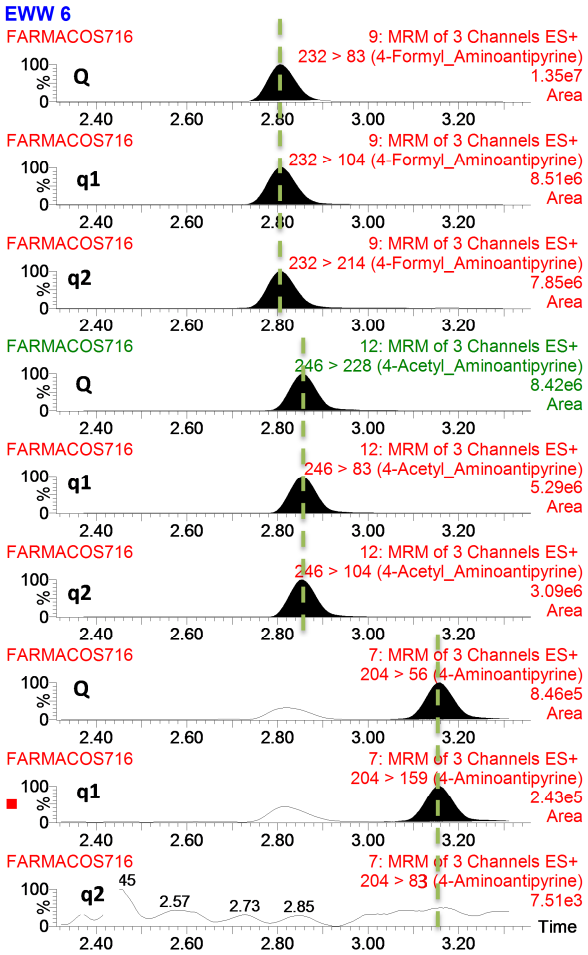

Figura 2.4 Cromatogramas UHPLC-MS/MS de los 3 metabolitos de la dipirona detectados en una muestra de agua residual de efluente.

CAPÍTULO 3

INVESTIGACIÓN DE LA PRESENCIA DE OMEPRAZOL EN AGUAS: ESTUDIO DE DEGRADACIÓN Y METABOLISMO

3.1 Introducción

3.2 Artículo Científico 2: *Investigating the presence of omeprazole in water by liquid chromatography coupled to low and high resolution mass spectrometry: Degradation experiments.* Journal of Mass Spectrometry, 48 (2013) 1091-1100

3.3 Artículo Científico 3: *Identification on new omeprazole metabolites in wastewater and surface water.* Science of the Total Environment, 468 (2014) 706-714

3.4 Artículo Científico 4: *Investigation of omeprazole and venlafaxine metabolites in wastewater making use of high resolution mass spectrometry.* Artículo en proceso

3.5 Discusión de los resultados

3.1 Introducción

Como se ha visto en los capítulos anteriores, la presencia de fármacos en aguas es un tema que está despertando gran interés en los últimos años. La mayoría de estudios reportados centran su atención en un grupo seleccionado de compuestos, haciendo uso de métodos multi-residuos desarrollados para este fin, típicamente usando LC-MS/MS QqQ. Sin embargo, el número de trabajos que se centran en un único y problemático analito es muy escaso. Éste es el caso del omeprazol, que pese a ser uno de los fármacos más consumidos en el mundo (Gómez-Malato, 2007; Ortiz de García, 2013), apenas se detecta en las aguas (Ternes, 2001; van Nujis, 2010; Sousa, 2011; Hernando, 2007; Valcárcel, 2011).

El omeprazol (5-metoxi-2-[(4-metoxi-3,5-dimetil-piridin-2-il) metilsulfinil]-3H-benzimidazol, **Figura 3.1**) pertenece a la familia de los inhibidores de la bomba de protones. Estos son un grupo de medicamentos cuya acción principal es la reducción pronunciada y duradera de ácido en el estómago. El omeprazol se une a la bomba de protones en la célula parietal gástrica, inhibiendo el transporte final de H^+ al lumen gástrico.

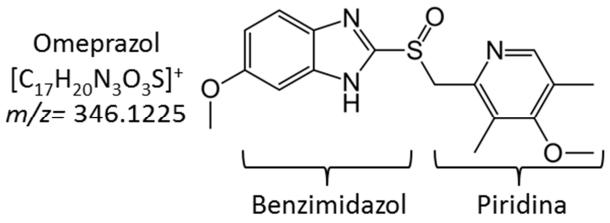

Figura 3.1 Estructura del omeprazol.

Desde un punto de vista químico, el omeprazol se caracteriza por ser protolíticamente activo en dos reacciones. Puede aceptar un protón en el nitrógeno del anillo piridínico (pKa=4), o bien se puede liberar un protón del grupo NH del benzimidazol (pKa=8.7). A valores de pH intermedios entre dichos pKas, predomina la forma neutra que es la única forma reactiva. El omeprazol es un sulfóxido y un compuesto quiral, siendo el átomo de azufre el centro estereogénico. De este modo, se muestra como una mezcla racémica de sus dos enantiómeros individuales, R-omeprazol y S-omeprazol, teniendo este último el nombre genérico de esomeprazol.

El proceso de metabolización de fármacos se produce generalmente 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, introduciendo 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 glucuronidación, en la que el ácido glucurónico se une a los grupos reactivos del compuesto, tales como grupos fenol, carboxilo, tiol 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, favoreciendo, por tanto, su eliminación a través de la

orina o 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 según el compuesto. 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.

Al pasar por las estaciones depuradoras de agua residual, tanto fármacos como metabolitos pueden sufrir transformaciones biológicas y/o físico-químicas desde el momento de su excreción, hasta alcanzar el medio ambiente. Una vez presentes en las aguas medioambientales (por ejemplo, aguas superficiales), los procesos de transformación pueden continuar dando lugar a una amplia variedad de TPs. Dependiendo de cuál sea la procedencia de las aguas (e. g. superficial, subterránea, residual) y del proceso, natural o forzado, al que se vean sometidas (hidrólisis, biodegradación, cloración, foto-degradación…), los productos que se generarán serán diferentes, originándose, en algunos casos, compuestos que pueden presentar un perfil ambiental y tóxico distinto al del compuesto de origen (**Figura 3.2**).

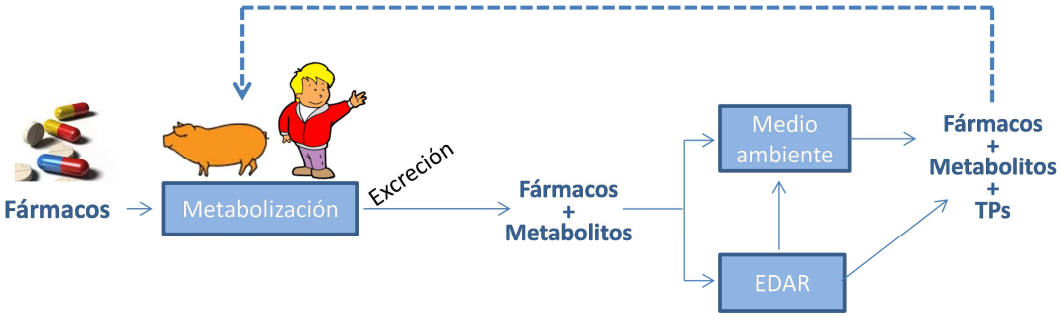

Figura 3.2 Vías de transformación de los fármacos desde su consumo hasta su llegada al medio ambiente.

En general, la información disponible acerca de la presencia de metabolitos/TPs en el medio ambiente es escasa. Prueba de ello es que tan sólo alrededor de 30 subproductos (metabolitos y TPs) se han estudiado en los trabajos publicados (Mompelat, 2009). La importancia de su presencia en el medio ambiente todavía no está bien investigada, pero para proteger la calidad del agua, la Agencia Europea de Medicamentos (*European Medicines Agency*, EMA) y la Agencia de Alimentos y Medicamentos de Estados Unidos (*Food and Drug Administration U.S.*, FDA) establecen que, antes de comercializar un nuevo fármaco se debe valorar su riesgo ambiental. Eso implica, entre otros aspectos, conocer el metabolismo del producto y los porcentajes de excreción del compuesto inalterado 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 tampoco sobre su persistencia en el medio ambiente. Algunos metabolitos y productos de transformación pueden ser peligrosos, incluso más que el compuesto de partida (Kostopoulou, 2008; Farré, 2008), por lo que, deberían ser controlados e incluidos en futuras metodologías analíticas desarrolladas.

La cromatografía líquida combinada con espectrometría de masas (LC-MS) se considera la técnica analítica más adecuada para la detección de fármacos, metabolitos o productos de transformación -la mayoría de ellos de alta polaridad- en matrices complejas, como pueden ser las medioambientales. Gracias a su robustez y sensibilidad esta tecnología permite determinar compuestos a niveles de ppb e incluso de ppt (Farré, 2008).

El analizador TOF ofrece la posibilidad de investigar la presencia de un compuesto *a posteriori*, es decir, una vez realizados los análisis y adquirido los datos de masas.

Haciendo uso de la terminología propuesta por nuestro grupo de investigación, a este modo de trabajo se le denomina análisis *"post-target"*. Resulta adecuado para el *screening* e identificación de un amplio número de contaminantes, para la confirmación de posibles muestras positivas analizadas mediante otras técnicas y para 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"* usando técnicas de HRMS es la posibilidad de realizar un análisis retrospectivo. Éste permite detectar e identificar otros compuestos de interés, que en un principio no se habían considerado, revisando los cromatogramas y espectros de masas adquiridos en muestras previamente analizadas. Para ello, los iones con una *m*/*z* específica se extraen del cromatograma TIC (*Total Ion Current*), generando los cromatogramas XIC (*eXtracted Ion Cromatogram*) con ventanas de masa estrecha (*narrow-mass window*), generalmente de 10 o 20 mDa. La presencia del compuesto en la muestra genera el correspondiente pico cromatográfico, que debe ser posteriormente examinado con detalle para comprobar si se trata del compuesto de interés o de un falso positivo. Esto se consigue mediante la información aportada sobre iones fragmentos presentes en la muestra, su compatibilidad con la estructura química del candidato, mediante los errores de masa obtenidos y mediante el perfil isotópico observado. Este modo de trabajo permite, 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 información adicional relevante sobre los iones fragmento del compuesto. En el QTOF, la presencia de un filtro cuadrupolar previo permite realizar experiencias en modo MS/MS. También permite 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, y por lo general, en los espectros LE predominan los iones correspondientes a las moléculas (des)protonadas (en ocasiones, sus aductos sodiados o amoniados). A alta energía de colisión, en cambio, las moléculas se fragmentan en la celda de colisión dando lugar a sus respectivos iones fragmento. 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 única inyección. Asimismo, la adquisición en MS^E proporciona información de la distribución isotópica de los iones fragmento, conservando la información de los aductos y dímeros, en su caso.

La información aportada por equipos de HRMS, como QTOF, es muy valiosa en investigaciones medioambientales por la cantidad de información relevante que aportan sobre la composición de la muestra. Sin embargo, presentan una importante limitación en comparación con los de triple cuadrupolo, que es su menor sensibilidad, lo que puede complicar la detección de ciertos compuestos cuando están presentes a niveles de sub-ppb. Por este motivo, los equipos LC-MS/MS con analizador QqQ trabajando en modo *Selected Reaction Monitoring* (SRM), constituyen la herramienta analítica ideal para la detección y cuantificación de fármacos en el medio ambiente. A su elevada sensibilidad, se une también su excelente

selectividad, dado que durante todo el tiempo del análisis se están adquiriendo varias transiciones específicas. Por estos motivos el triple cuadrupolo en modo SRM en una herramienta muy valiosa, tanto para la cuantificación como para la confirmación de los compuestos.

Los tres trabajos que se incluyen en el presente capítulo, surgieron tras valorar la problemática detección del omeprazol en las aguas. Como se ha comentado anteriormente, si se considera el alto consumo de omeprazol y que su máximo porcentaje de excreción reportado es del 30% (Ortiz de García, 2013), se podría esperar la presencia de este compuesto en aguas residuales o incluso en ambientales. Sin embargo, este fármaco es raramente detectado en las aguas tal como reportan varios artículos científicos (Valcárcel, 2011; Sousa, 2011; Castiglioni, 2006; Gracia-Lor, 2014; van Nuijs, 2010). Con esta información, corroborada con nuestra propia experiencia, surgió la primera hipótesis, que apuntaba a la degradación del omeprazol tras los tratamientos en las estaciones depuradoras, como la principal causa de la ausencia del fármaco en las aguas. Surgió así el trabajo detallado en el *Artículo Científico 2*.

En este artículo se llevaron a cabo experimentos de degradación (hidrólisis, cloración y foto-degradación) en condiciones controladas de laboratorio. Tras los análisis por UHPLC-QTOF en modo MS^E se obtuvieron diferentes productos de transformación, que se intentaron elucidar utilizando la información obtenida en los espectros de masas a baja y alta energía de colisión. Con el fin de investigar la presencia en las aguas de los OTPs (*Omeprazole Transformation Products*) identificados en experiencias de laboratorio, se realizó un análisis retrospectivo en muestras residuales y superficiales previamente analizadas en nuestro laboratorio. Adicionalmente, también se investigaron dichos OTPs en nuevos análisis realizados

mediante LC-MS/MS con analizador de triple cuadrupolo, para facilitar la detección de estos compuestos a muy bajos niveles de concentración, y de paso confirmar la detección previa realizada mediante QTOF MS.

No completamente satisfechos con los resultados obtenidos, pues los TPs detectados en muestras reales eran poco abundantes, se planteó una segunda hipótesis. En ella se consideró que la ausencia de omeprazol en las aguas se debía a la elevada metabolización del fármaco en el cuerpo humano, excretándolo en mayor medida como diversos metabolitos. En esta hipótesis se trataba de focalizar la búsqueda no sólo en el omeprazol, sino también en sus metabolitos. Tras la falta de bibliografía detallada y contrastada sobre el metabolismo del omeprazol, se planteó el trabajo que está descrito en el *Artículo Científico 3*.

En dicho estudio se llevó a cabo la investigación de metabolitos de omeprazol en orina humana haciendo uso de UHPLC-QTOF MS. Tras la identificación de distintos metabolitos en orinas de tres voluntarios, se investigó la presencia de estos metabolitos en un notable número de aguas superficiales y residuales mediante análisis realizados por LC-QTOF MS y LC-QqQ MS.

Por último, el trabajo reportado en el *Artículo Científico 4* surgió a raíz de una estancia de la doctoranda en el ***Instituto di Ricerche Farmacologiche Mario Negri*** en Milán (Italia). Dicha estancia fue supervisada por el Profesor Ettore Zuccato y se llevó a cabo en los meses de Febrero y Marzo de 2014. El enfoque del trabajo surgió tras la falta de detección del omeprazol en aguas italianas. Se planteó, entonces, centrar la búsqueda de residuos de omeprazol mediante el análisis de sus metabolitos (reportados en el *Artículo Científico 3*) en lugar del fármaco original. Con este trabajo se pretende demostrar la utilidad de los estudios previamente desarrollados en nuestro laboratorio, en un estudio aplicado realizado en otro país,

con otras aguas y con otro equipo instrumental diferente (Orbitrap). En el *Artículo Científico 4* se investiga la presencia de omeprazol y venlafaxina, así como sus metabolitos, en aguas residuales urbanas tomada en Italia. Para ello, se analizaron 30 muestras de influente (IWW) procedentes de diez ciudades italianas mediante HPLC-Orbitrap MS.

3.2 Artículo Científico 2

Research article

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Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry: degradation experiments

C. Boix,[a] M. Ibáñez,[a] J. V. Sancho,[a] W. M. A. Niessen[b] and F. Hernández[a]*

Omeprazole is one of the most consumed pharmaceuticals around the world. However, this compound is scarcely detected in urban wastewater and surface water. The absence of this pharmaceutical in the aquatic ecosystem might be due to its degradation in wastewater treatment plants, as well as in receiving water. In this work, different laboratory-controlled degradation experiments have been carried out on surface water in order to elucidate generated omeprazole transformation products (TPs). Surface water spiked with omeprazole was subjected to hydrolysis, photo-degradation under both sunlight and ultraviolet radiation and chlorination. Analyses by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC–QTOF MS) permitted identification of up to 17 omeprazole TPs. In a subsequent step, the TPs identified were sought in surface water and urban wastewater by LC–QTOF MS and by LC coupled to tandem mass spectrometry with triple quadrupole. The parent omeprazole was not detected in any of the samples, but four TPs were found in several water samples. The most frequently detected compound was OTP 5 (omeprazole sulfide), which might be a reasonable candidate to be included in monitoring programs rather than the parent omeprazole. Copyright © 2013 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: omeprazole; transformation/degradation products; ultra-high-performance liquid chromatography; time-of-flight mass spectrometry; triple quadrupole mass spectrometry; surface water; urban wastewater

Introduction

Large amounts of pharmaceuticals are used every year around the world, being omeprazole one of the most consumed.[1,2] This compound belongs to the proton pump inhibitor group, whose main action is a pronounced and long-lasting reduction of gastric acid production. Omeprazole being a racemate containing a mixture of (*R*)- and (*S*)-enantiomers. This pharmaceutical undergoes *in vivo* a chiral shift, which converts the inactive (*R*)-enantiomer to the active (*S*)-enantiomer, doubling the concentration of the active form. As other drugs, omeprazole can reach the aquatic environment through urine excretion and improper disposal. Therefore, one would expect to find omeprazole in wastewater and even in surface water, if an incomplete removal occurs in wastewater treatment plants (WWTPs), as already observed for other pharmaceuticals.[2,3] However, omeprazole is not usually detected when analyzing this type of samples,[4–8] and only a few papers have reported the presence of this compound in wastewater.[1,9,10]

Modern multi-class methods applied for the determination of omeprazole and other polar pharmaceuticals are mostly based on liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ)[4,5,7,9,11] or ion trap (IT)[6,10] analyzers. The presence of omeprazole in environmental samples has also been investigated by LC coupled to time-of-flight mass spectrometry (TOF MS).[1,12] LC–TOF MS is a valuable tool for screening pharmaceuticals and their metabolites/transformation products (TPs) in water because of its accurate mass measurements, high resolving power and high full-spectrum sensitivity.[13–17] Recoveries reported in most LC–MS/MS methods for omeprazole in spiked water are satisfactory (between 77% and 120%) provided that an appropriate internal standard is used for matrix effects correction.[4–7,9] Therefore, the absence of omeprazole in the water samples seems not to be related to analytical issues but to human metabolism and/or degradation/transformation of this compound in the aquatic environment. Thus, not only omeprazole but also its related compounds, such as metabolites and/or TPs, should be investigated in water as they might be appropriate target analytes when investigating the impact of omeprazole in the aquatic environment.

Very few articles have been reported dealing with the degradation of omeprazole in water. DellaGreca *et al.*[18] investigated the chemical and photochemical behavior of omeprazole in the aquatic environment, suggesting several structures of possible TPs after the analysis by ^{1}H NMR and TLC. Omeprazole instability in acid-aqueous conditions[1,11,18,19] has also been reported.

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INVESTIGATING THE PRESENCE OF OMEPRAZOLE IN WATERS BY LIQUID CHROMATOGRAPHY COUPLED TO LOW AND HIGH RESOLUTION MASS SPECTROMETRY: DEGRADATION EXPERIMENTS

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ABSTRACT

Omeprazole is one of the most consumed pharmaceuticals around the world. However, this compound is scarcely detected in urban wastewater and surface water. The absence of this pharmaceutical in the aquatic ecosystem might be due to its degradation in wastewater treatment plants, as well as in receiving water. In this work, different laboratory-controlled degradation experiments have been carried out on surface water in order to elucidate generated omeprazole transformation products (TPs). Surface water spiked with omeprazole was subjected to hydrolysis, photo-degradation under both sunlight and ultraviolet radiation, and chlorination. Analyses by liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QTOF MS) permitted identification of up to 17 omeprazole TPs. In a subsequent step, the TPs identified were sought in surface water (SW) and urban wastewater (WW) by LC-QTOF MS and by LC-MS/MS with triple quadrupole. Parent omeprazole was not detected in any of the samples but four TPs were found in several water samples. The most frequently detected compound was OTP5 (omeprazole sulfide), which might be a reasonable candidate to be included in monitoring programs rather than parent omeprazole.

Keywords

Omeprazole, transformation/degradation products, ultra-high-performance liquid chromatography, time-of-flight mass spectrometry, triple quadrupole mass spectrometry, surface water, urban wastewater.

1. INTRODUCTION

Large amounts of pharmaceuticals are used every year around the world, being omeprazole one of the most consumed [1,2]. This compound belongs to the proton pump inhibitor group, whose main action is a pronounced and long-lasting reduction of gastric acid production. Omeprazole is a racemate containing a mixture of (R)- and (S)-enantiomers. This pharmaceutical undergoes *in vivo* a chiral shift, which converts the inactive (R)-enantiomer to the active (S)-enantiomer, doubling the concentration of the active form. As other drugs, omeprazole can reach the aquatic environment through urine excretion and improper disposal. Therefore, one would expect to find omeprazole in wastewater and even in surface water, if an incomplete removal occurs in wastewater treatment plants (WWTPs), as already observed for other pharmaceuticals [2,3]. However, omeprazole is not usually detected when analyzing this type of samples [4-8], and only a few papers have reported the presence of this compound in wastewater [1,9,10].

Modern multi-class methods applied for the determination of omeprazole and other polar pharmaceuticals are mostly based on liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) [4,5,7,9,11] or ion trap (IT) [6,10] analyzers. The presence of omeprazole in environmental samples has also been investigated by LC coupled to time-of-flight mass spectrometry (TOF MS) [1,12]. LC-TOF MS is a valuable tool for screening pharmaceuticals and their metabolites/TPs in water due to the accurate mass measurements, high resolving power and high full-spectrum sensitivity [13-17]. Recoveries reported in most LC-MS/MS methods for omeprazole in spiked water are satisfactory (between 77 and 120%) provided that an appropriate internal standard is used for matrix effects correction [4-7,9]. Therefore, the absence of omeprazole in the water samples seems not to be related to analytical issues but to human metabolism and/or degradation/transformation of this compound in the aquatic environment. Thus, not only omeprazole but also its related-compounds, such as metabolites and/or transformation products (TPs), should be investigated in water as they might be appropriate target analytes when investigating the impact of omeprazole in the aquatic environment.

Very few articles have been reported dealing with the degradation of omeprazole in water. DellaGreca et al. [18] investigated the chemical and photochemical behavior of omeprazole in the aquatic environment, suggesting several structures of possible TPs after the analysis by ^{1}H NMR and TLC. Omeprazole instability in acid-aqueous conditions [1,11,18,19] has also been reported.

On the basis that omeprazole is one of the most consumed pharmaceuticals but it is scarcely detected in water samples, the goal of this work is to perform a detailed study of its degradation/transformation in water under laboratory-controlled conditions. Different degradation assays (hydrolysis, photo-degradation and chlorination) have been performed and plausible identifications of the omeprazole TPs have been obtained with LC-QTOF MS. After that, a retrospective analysis has been performed in wastewater and surface water samples (previously analyzed by QTOF for screening of pharmaceuticals), searching for those TPs discovered in the degradation experiments. The same samples have also been re-analyzed using LC-MS/MS QqQ, where enhanced instrumental sensitivity was expected, to facilitate the TPs detection at low concentration levels. The investigation on omeprazole urinary human metabolism and on the presence of its metabolites in environmental and wastewater can be found elsewhere [20].

2. EXPERIMENTAL

2.1. Reagents and chemicals

(For further details, see Supplementary Information, SI).

2.2. Instrumentation

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration–TOF mass spectrometer (Q-TOF Premier, Waters Micromass, Manchester, UK), using an orthogonal Z-spray electrospray ionization (ESI) interface operating in positive and negative ion modes. Accurate-mass full-spectrum data were acquired simultaneously at low (LE) and high (HE) collision energy (MS^E mode). Thus, relevant information on the (de)protonated molecule and on its main fragments could be obtained in just one sample injection *(for further details, see SI).*

A Waters Acquity UPLC system was interfaced to a triple quadrupole mass spectrometer (TQD, Waters) with an orthogonal Z-spray ESI interface. Capillary voltages of 3.5 and -3.0 kV were used in positive and negative ion mode, respectively (*for further details, see SI).*

2.3. Degradation experiments

Four different degradation assays were carried out under laboratory-controlled conditions in attempts to simulate some of the probable processes occurring in the aquatic environment and in a WWTP. Hydrolysis, photo-degradation, and chlorination experiments were performed to study the degradation products in surface water spiked at 0.5 μg mL^{-1} with omeprazole. The main characteristics of this water are shown in Table 1S1. The general methodology applied in this work has been previously published [21-23].

Hydrolysis: 50 mL spiked surface water was maintained at room temperature in darkness. Then, 2-mL aliquots were sampled at different time intervals (0-17 days) to study omeprazole stability in aqueous solution. The reaction was stopped by freezing the collected aliquots at –18 ºC. In order to take into account a possible microbiological degradation in surface water, the hydrolysis assays were also performed in drinking water.

Photo-degradation (sunlight and ultraviolet light): Sunlight experiments were carried out using a solar simulated Suntest XL (Atlas, Linsengericht, Germany) equipped with a xenon arc lamp as radiation source, and a solar light filter restricting wavelength below 290 nm. The radiation intensity was 500 W m^{-2} and the light dose per hour of irradiation was 1.8 MJ h^{-1}. Suntest assays were performed in 250 mL quartz glass vessels at 25 ºC. Under these conditions, 90 irradiation hours simulated the natural sun light of approximately 15 days (Dose: 288 MJ m^{-2}).

Ultraviolet experiments were performed using a mercury vapour lamp with its main output at 254 nm. The distance from the lamp to the surface of the samples was approximately 15 cm. Irradiation was carried out in a fume hood at room temperature over a period about 60 hours under constant stirring of the samples.

In both type of photo-degradation experiments, 250-mL of surface water samples spiked with omeprazole at 0.5 µg mL^{-1} were used. Aliquots of 2-mL were sampled at different times.

Chlorination: The experiments were carried out by adding 30 µL of commercial sodium hypochlorite 1% w/v solution to 50-mL surface water spiked at 0.5 µg mL^{-1} with omeprazole. The reaction mixture was maintained at room temperature in darkness, and 2-mL water aliquots were taken at different time intervals (0-4 days).

In all cases, after collecting the water aliquots, the reaction was stopped by immediately freezing at –18 ºC. In all experiments, non-spiked surface water samples were also subjected to the same conditions and used as control samples.

After degradation experiments 50 µL of water aliquots were directly injected into the LC-QTOF MS.

2.4. Retrospective QTOF MS analysis of water samples

25 wastewater samples (15 influents and 10 effluents) were collected from three WWTPs of the Valencian Community (Eastern Spain) whose anonymity must be respected, from June 2008 to December 2010. Additionally, 27 surface waters were sampled from several points located in the same area in October 2010. All water samples had been subjected to solid phase extraction [9,24], and analyzed by LC-QTOF MS for other research purposes. In this study, the accurate-mass full-spectrum data were retrospectively re-processed to search for omeprazole TPs without the need of additional analysis.

2.5. Data processing

QTOF MS data were processed using MetaboLynx XS and ChromaLynx XS application managers (Micromass v 4.1). Regarding data from triple quadrupole instrument, TargetLynx (also from Micromass v 4.1) was used.

Data processing using MetaboLynx XS

MetaboLynx XS software was used to process data obtained from degradation studies. This software compares eXtracted Ion Chromatograms (XICs) of a positive/degraded sample to a control sample for detecting, identifying and reporting differential ions/chromatographic peaks which would correspond, in principle, to transformation products. In previous studies [25-27], MetaboLynx XS proved to be highly useful for the investigation of both expected and unexpected metabolites/TPs.

Acquisitions were performed in centroid, in both positive and negative ion mode. For all compounds detected by MetaboLynx, the accurate mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Then, possible elemental compositions were calculated using the MassLynx elemental composition calculator with a maximum deviation of 2 mDa from the measured accurate mass. The maximum and minimum parameters were restricted considering the elemental composition and structure of omeprazole ($C_{17}H_{19}N_3O_3S$) as follows: C 0–25, H 0–40, N 0–4, O 0–10 and S 0–2. In chlorination experiments, the number of chlorine atoms was selected based on the observed isotopic pattern. The applied double-bond equivalent

(DBE) filter was set between –0.5 and 50. In those cases where more than one elemental composition was obtained, neutral losses were investigated in both HE and LE functions, trying to reduce the number of plausible elemental compositions. To calculate the elemental composition of fragment ions, parameters settings were restricted as a function of the calculated elemental composition of the (de)protonated molecule, while for neutral losses no restrictions were applied. Additionally, the option "even-electrons ions only" was selected for the precursor ion, and "odd- and even-electrons ions" for the fragment ions.

Data processing using ChromaLynx XS

QTOF MS data were processed by ChromaLynx XS in target mode for retrospective analysis of omeprazole TPs in waters. This software permits application of a "post-target" processing method based on selected theoretical exact masses (target list) that permits a rapid and simple reviewing by cataloguing analytes, as functions of mass error and retention time deviation [24,27,28].

Data processing using TargetLynx XS

TargetLynx software was employed for automatically processing data from triple quadrupole analysis. For confirmation of positive findings, the acquisition of two SRM transitions per compound together with the agreement in both retention time and Q/q ratio deviation were required [29]. Reference Q/q ratios were obtained from TPs identified in degradation experiments as the ratio between the most abundant ion (Q, quantitative) and the other/s measured ion/s (q, confirmation).

3. RESULTS AND DISCUSSION

Figure 1 shows the structure of the most abundant product ions of omeprazole ($C_{17}H_{20}N_3O_3S^+$, *m/z* 346.1225). All product ions correspond to the pyridine ring containing fragment, except for *m/z* 149, which comes from the benzimidazole ring.

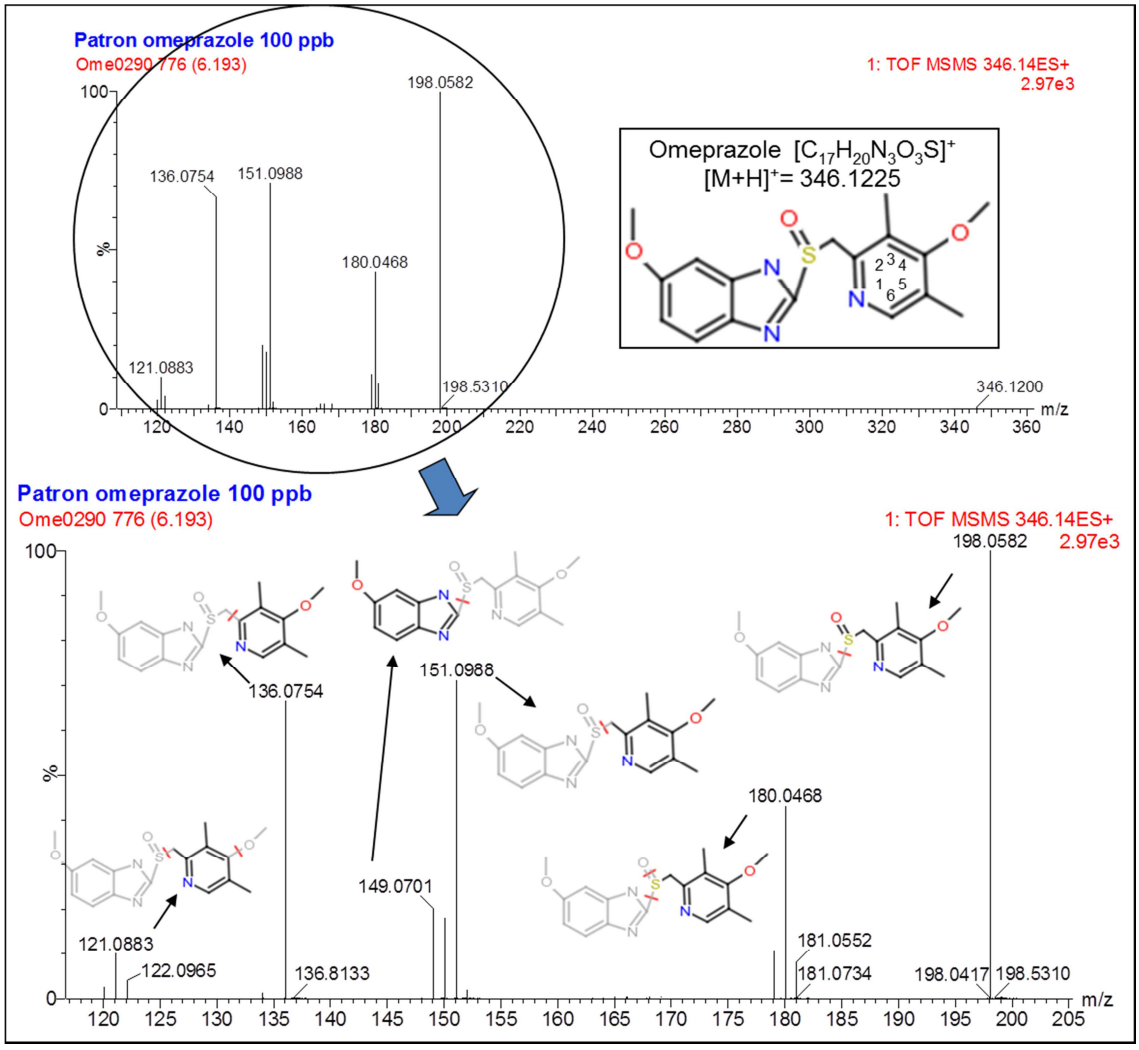

Figure 1. QTOF product ion spectrum of omeprazole and product ions structures.

3.1. Degradation experiments

The results obtained in degradation experiments are summarized in Table 1. It shows the elemental composition of the seventeen TPs detected together with their retention times and fragment ions. In general, mass errors were lower than 2 mDa, facilitating reliable elemental composition assignments.

Table 1. Hydrolysis, photo-degradation and chlorination TPs of omeprazole obtained by LC-ESI-QTOF MS, and their main fragment ions.

Compound	RT (min)	Elemental composition	Exp mass (*m/z*)	Mass Error (mDa)
Omeprazole	5.98	$C_{17}H_{20}N_3O_3S^+$	346.1211	-1.4
		$C_9H_{12}NO_2S^+$	198.0582	-0.7
		$C_9H_{10}NOS^+$	180.0468	-1.5
		$C_9H_{13}NO^{+\cdot}$	151.0988	-0.9
		$C_9H_{12}NO^+$	150.0921	0.2
		$C_8H_9N_2O^+$	149.0701	-1.4
		$C_8H_{10}NO^+$	136.0754	-0.8
		$C_8H_{11}N^{+\cdot}$	121.0883	-0.8
		$C_8H_{10}N^+$	120.0828	1.5
Hydrolysis				
OTP 1	1.93	$C_9H_{14}NO_2^+$	168.1030	0.5
		$C_9H_{12}NO^+$	150.0929	1.0
		$C_8H_{10}N^+$	120.0819	0.6
OTP 2	2.37	$C_9H_{12}NO_3^+$	182.0822	0.5
		$C_8H_{12}NO_2^+$	154.0873	0.5
		$C_8H_{10}NO^+$	136.0762	0.0
OTP 3	3.86	$C_{17}H_{18}N_3O_4^+$	328.1313	1.6
		$C_{17}H_{16}N_3O_3^+$	310.1178	-1.4
		$C_8H_{12}NO_2^+$	154.0849	-1.9
		$C_8H_{10}NO^+$	136.0767	0.5
		$C_7H_8N^+$	106.0649	-0.8
OTP 4	4.09	$C_{17}H_{20}N_3O_2^+$	298.1556	0.0
		$C_{15}H_{14}N_3O_2^+$	268.1104	1.8
		$C_{14}H_{14}N_3O^+$	240.1133	-0.4
		$C_9H_{12}NO^+$	150.0931	1.2
		$C_8H_{10}NO^+$	136.0763	0.1
		$C_8H_{10}N^+$	120.0807	-0.6
OTP 5	5.68	$C_{17}H_{20}N_3O_2S^+$	330.1271	-0.5
		$C_{17}H_{19}N_3O_2^{+*}$	297.1495	1.8
		$C_{16}H_{16}N_3O_2^{+**}$	282.1235	0.8
		$C_9H_{12}NOS^+$	182.0635	-0.5
		$C_9H_{12}NO^+$	150.0914	-0.5
		$C_8H_9N_2O^+$	149.0719	0.4
		$C_8H_{10}NO^+$	136.0760	-0.2
		$C_8H_{10}N^+$	120.0818	0.5

Table 1 (Cont.). Hydrolysis, photo-degradation and chlorination TPs of omeprazole obtained by LC-ESI-QTOF MS, and their main fragment ions.

Compound	RT (min)	Elemental composition	Exp. mass (*m/z*)	Mass Error (mDa)
Photo-degradation				
OTP 6	1.71	$C_9H_{12}NO_4^+$	198.0773	0.7
		$C_9H_{10}NO_3^+$	180.0672	1.1
		$C_8H_{10}NO^+$	136.0752	-1.0
		$C_7H_8NO^+$	122.0605	-0.1
OTP 7 -10	3.45 - 3.55 4.82 - 5.03	$C_8H_8N_3O_3^+$	194.0565	-0.1
Chlorination				
OTP 11 - 12	7.21 - 7.52	$C_{17}H_{17}N_3O_3Cl^+$	346.0942	-1.6
		$C_{17}H_{16}N_3O_3ClNa^+$	368.0595	-0.5
		$C_9H_{10}NO_2^+$	164.0700	-1.2
		$C_8H_{12}NO_2^+$	154.0870	0.2
		$C_8H_{10}NO^+$	136.0753	-0.9
		$C_7H_8N^+$	106.0654	-0.3
OTP 13	7.70	$C_{17}H_{18}N_3O_4SCl_2^+$	430.0405	1.0
		$C_{17}H_{17}N_3O_4SCl_2Na^+$	452.0207	-0.8
		$C_{17}H_{18}N_3O_2Cl_2^+$	366.0777	0.1
		$C_9H_{12}NO_3S^+$	214.0535	-0.3
		$C_9H_{12}NO_2^+$	166.0871	0.3
		$C_9H_{12}NO^+$	150.0907	-1.2
		$C_8H_{10}NO^+$	136.0746	-1.6
		$C_8H_{10}N^+$	120.0800	-1.3
OTP 14	8.23	$C_{17}H_{16}N_3O_3Cl_2^+$	380.0556	-1.3
		$C_{17}H_{15}N_3O_3Cl_2Na^+$	402.0382	-0.6
		$C_9H_{10}NO_2^+$	164.0696	-1.6
		$C_8H_{12}NO_2^+$	154.0863	-0.5
		$C_8H_{10}NO^+$	136.0754	-0.8
OTP 15 – 16	8.34 - 8.46	$C_{17}H_{17}N_3O_4SCl_3^+$	464.0010	0.5
		$C_{17}H_{16}N_3O_4SCl_3Na^+$	485.9833	0.8
		$C_{16}H_{15}N_3O_4SCl^+$	380.0487	1.5
		$C_{17}H_{16}N_3O_2Cl_2^+$	364.0620	0.0
		$C_{15}H_{10}N_3O_2Cl_2^+$	334.0139	-1.1
		$C_9H_{11}NOCl^+$	184.0545	1.6
		$C_9H_{12}NO_2^+$	166.0875	0.7
OTP 17	9.15	$C_{16}H_{14}N_3O_4SCl_2^+$	414.0081	-0.1
		$C_{16}H_{13}N_3O_4SCl_2Na^+$	435.9888	-1.4
		$C_{16}H_{14}N_3O_2Cl_2^+$	350.0458	-0.5
		$C_{15}H_{11}N_3O_2Cl^+$	300.0531	-0.9
		$C_{15}H_{13}N_3OCl^+$	286.0736	-1.1

* Loss of [·HS] radical

** Loss of [·CH_3] radical

Figure 2 shows the detection of an omeprazole TP (OTP 6) after 90 hours of Suntest experiments, using MetaboLynx software in the unexpected metabolite mode. The presence of a chromatographic peak was observed (Rt 1.71 min) in the sample (Figure 2a) which was absent in the blank control (Figure 2b). From the experimental accurate mass (or *m/z*) at *m/z* 198.0773 (mass error +0.7 mDa), an elemental composition of $C_9H_{12}NO_4^+$ was calculated. Two other proposed elemental compositions were not feasible as the type and number of chemical elements as well as DBEs were not compatible with the chemical structure of omeprazole. Similarly to this example, a plausible elemental composition was assigned for all TPs found and chemical structures were proposed.

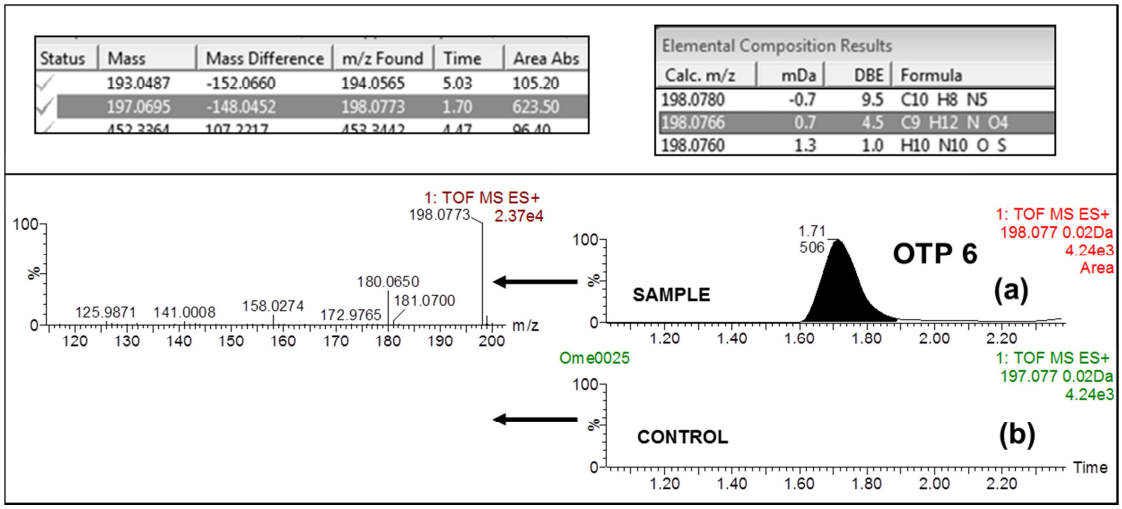

Figure 2. Detection of OTP 6 using MetaboLynx XS software. narrow-window eXtracted Ion Chromatogram (nw-XIC) at *m/z* 198.0766 (0.02 Da mass window width) for (a) analyte and (b) control, after 90 hours of suntest photo-degradation.

The chemical structures proposed for TPs discovered in hydrolysis and photo-degradation experiments performed in surface water are depicted in Figure 3. In the ***hydrolysis*** experiments, five degradation products were detected (OTPs 1-5) (Figure 3a). OTPs 1 and 2 corresponded to the omeprazole pyridine ring with one or two additional oxygen atoms, respectively. The three remaining TPs would result from the loss of the sulfur atom plus hydroxylation in the pyridine ring (OTP 3), the loss of sulfoxide group (OTP 4), and the loss of one oxygen atom from the omeprazole structure (OTP 5). The loss of sulfur

atom from the middle of the molecule is explained in more detail in [20]. In order to take into account a possible microbiological degradation, the hydrolysis assay was also performed in drinking water matrix. All hydrolysis TPs were also detected in drinking water, although OTP 1 was less abundant than in surface water. It seems that the formation of the five observed TPs was due to hydrolysis more than to microbiological degradation from the organic matter present in surface water.

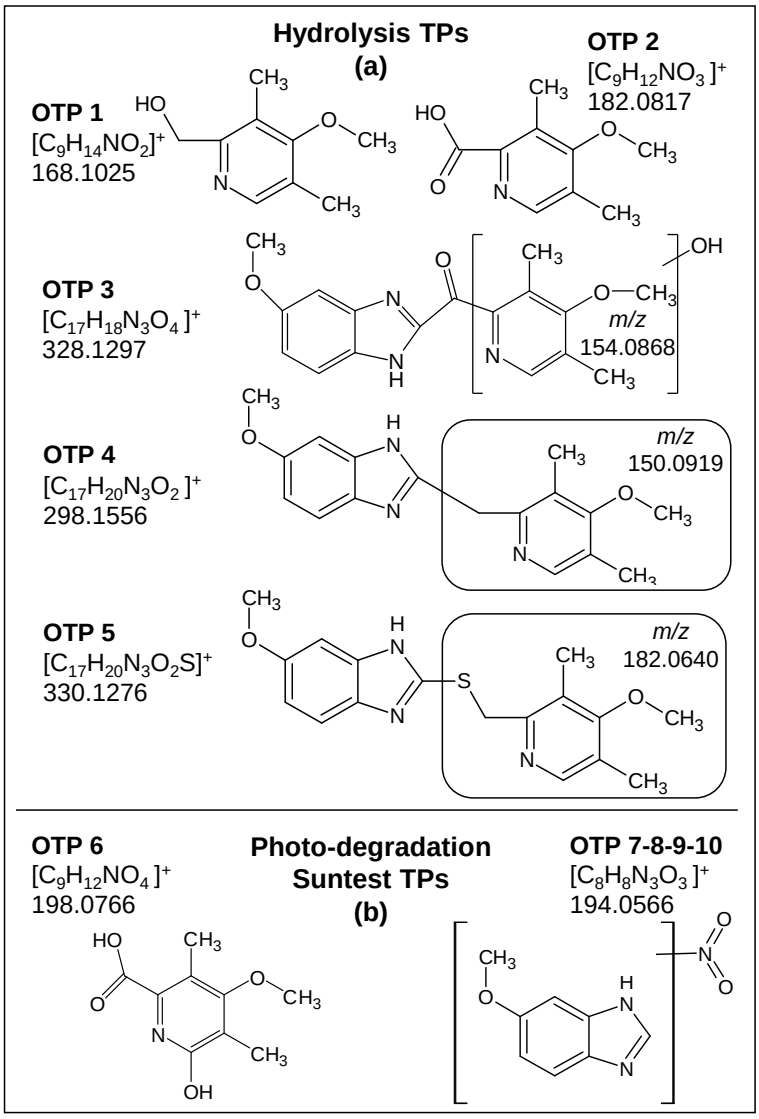

Figure 3**.** Proposed structures for omeprazole TPs, obtained after (a) hydrolysis (OTPs 1-5) and (b) suntest photo-degradation (OTPs 6-10).

As an illustrative example of the elucidation process, Figure 4 shows QTOF MS data obtained for OTP 5, which was afterwards identified in "real-world" water samples (sections 3.2 and 3.3). Up to our knowledge, this compound has not been previously reported in the literature. According to its accurate mass (*m/z* 330.1271, Figure 4a), the elemental composition for the protonated molecule was assigned to $C_{17}H_{20}N_3O_2S^+$ (ΔmDa=–0.5), which would imply the loss of one oxygen atom from the omeprazole molecule. The fragment ions at *m/z* 297.1495 ($C_{17}H_{19}N_3O_2^{+\bullet}$, ΔmDa=-1.8) and *m/z* 282.1235 ($C_{16}H_{16}N_3O_2^+$, ΔmDa=+0.8) would correspond to thiol [$^{\bullet}SH$] and subsequent methyl [$^{\bullet}CH_3$] radical losses, respectively, being related with the presence of a sulfide group [20]. Thus, we suggest an oxygen loss from the sulfur atom in the original omeprazole structure, which is in accordance with previous literature [18]. If so, the fragment ions *m/z* 150.0914 ($C_9H_{12}NO^+$, ΔmDa=-0.5), 149.0719 ($C_8H_9N_2O^+$, ΔmDa=0.4), 136.0760 ($C_8H_{10}NO^+$, ΔmDa=-0.2) and 120.0818 ($C_8H_{10}N^+$, ΔmDa=0.5), observed for omeprazole, should be observed in OTP 5 too, and indeed they are (see Figure 1 and Figure 4b). Regarding the fragment ion *m/z* 198.0589 ($C_9H_{12}NO_2S^+$, Figure 1) observed in the omeprazole spectrum, OTP 5 presents a fragment ion at *m/z* 182.0635 instead, corresponding to $C_9H_{12}NOS^+$ (see Figure 4c).

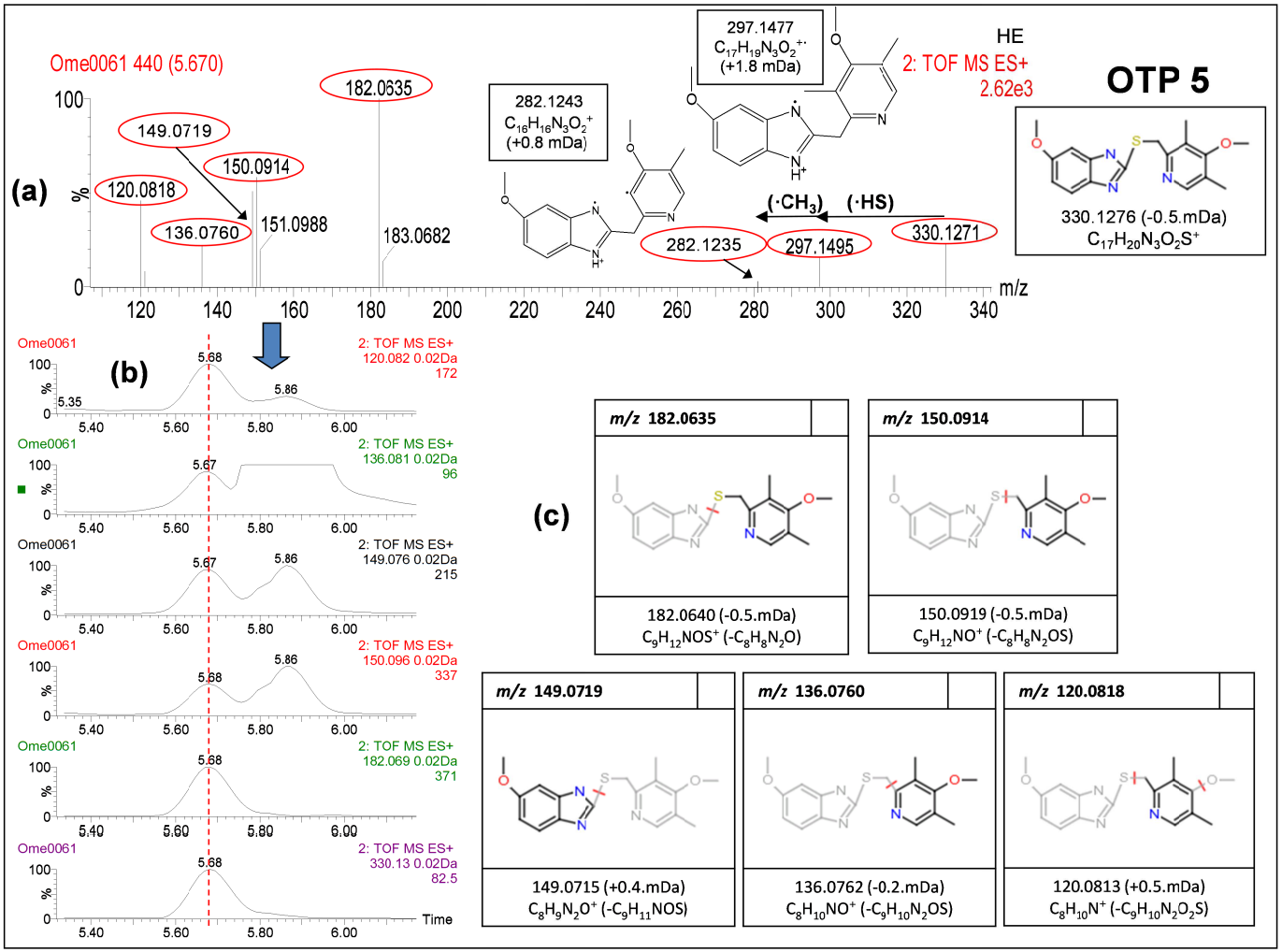

Figure 4. Detection and identification of OTP 5 by LC-QTOF MS (MS^E) obtained in the hydrolysis assay (a) HE spectrum and justification of some fragment ions, (b) XICs at 0.02 Da mass width window for different fragment ions observed in HE function, and (c) proposed structures for low *m/z* fragment ions of OTP 5.

In relation to ***photo-degradation*** experiments with UV lamp, omeprazole was observed to be quickly degraded, but no TPs could be detected. This is in agreement with previous literature, as UV has been reported for some compound as an effective process of disinfection, removing the parent compound, and generating few or none persistent TPs [23, 30]. However, in the Suntest experiments (Figure 3b) up to five TPs were found (OTPs 6-10). OTP 6 (*m/z* 198.0766, $C_9H_{12}NO_4^+$) would correspond to the modified pyridine part of omeprazole (observed at *m/z* 150.0919, $C_9H_{12}NO^+$ in the MS/MS spectrum of omeprazole) plus three additional oxygen atoms. OTPs 7–10 were isomeric compounds (*m/z* 194.0566, $C_8H_8N_3O_3^{+)}$ eluting at different retention times (3.45, 3.55, 4.82 and 5.03 min, respectively). These isomers could be related with the benzimidazole part of omeprazole (observed in the product ion spectrum of omeprazole as the product ion at *m/z* 149.0715, $C_8H_9N_2O^+$) and nitrated at different positions for each isomer. The NO_2 group could easily be introduced, because the photo-degradation experiments were performed in surface water from the Mijares River (Castellón province), where relatively high nitrate concentrations are common as a result of the wide use of fertilizers in this agricultural area (Table 1SI) [23, 26]. Nevertheless, it is difficult to predict where the nitration takes place for each isomer, as no information about fragmentation was available in these cases, even after performing MS/MS experiments.

The last assay performed was ***chlorination***, which generated seven TPs (OTPs 11-17). Figure 5a shows the chromatographic peaks obtained in the aliquot collected after 5 min. of chlorination. These compounds were less polar than omeprazole as they eluted later than the parent (5.98 min). Figure 5b shows the proposed chemical structures for these TPs, which would result from hydroxylation, oxidation and/or chlorination (between 1 and 3 chlorine atoms) of omeprazole.

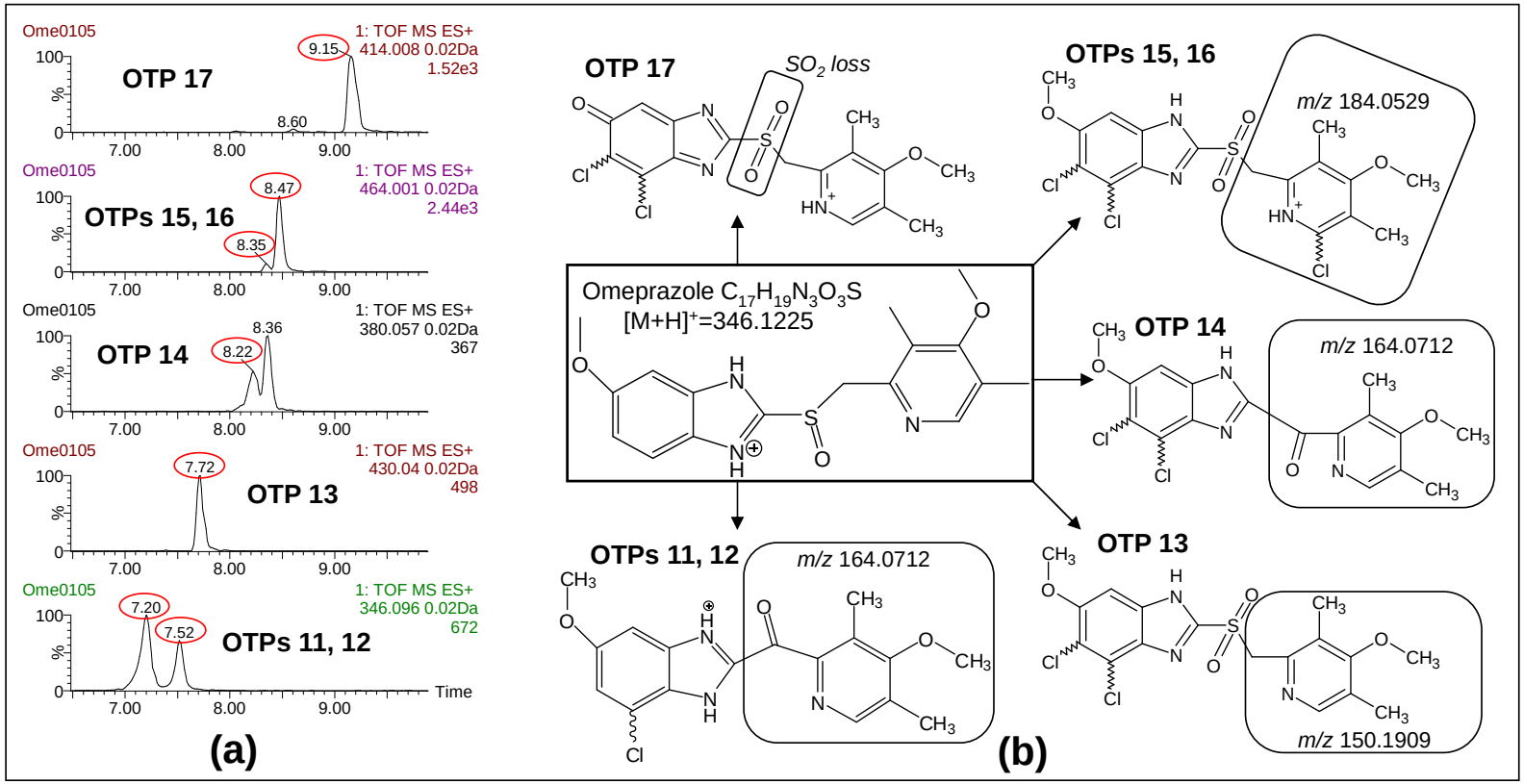

Figure 5. Proposed structures for TPs obtained after omeprazole chlorination (OTPs 11-17). (a) nw-XICs at 0.02 Da mass width window for each OTP and (b) suggested structures.

Retrospective analysis of surface water and wastewater by LC-QTOF MS

Analyses by QTOF MS make feasible to perform a retrospective evaluation of data at any time later on, due to the availability of untargeted accurate-mass full-spectrum data provided such compound has passed the sample preparation, chromatographic separation and ionization process [13].

In order to test whether the parent omeprazole or their TPs were present, a retrospective analysis was performed. For this purpose, 25 wastewater and 27 surface water samples, previously analyzed by LC-QTOF MS, were re-processed using ChromaLynx XS software. The database of the compounds investigated contained the elemental composition, fragment ions and retention time (±0.35 min) of the 17 omeprazole TPs resulting from degradation experiments and of the parent omeprazole too (Table 2).

The results of the retrospective analysis are shown in Table 3. It is worth noting that parent omeprazole was not found in any water samples. On the contrary, OTPs 4 and 5 were detected in several effluent wastewaters and surface waters based on the presence of their accurate-mass protonated molecules (LE function). However, their specific fragments were not clearly observed in the HE function, so their unequivocal identification was unfeasible, although the accurate mass of the protonated molecule together with the retention time strongly suggested that the compounds detected were in fact OTP 4 and OTP 5. It seems that the low concentration levels of these compounds in the samples required more sensitive analytical techniques to confirm their presence. Therefore, LC-MS/MS with triple quadrupole was used for complementary analysis.

Table 2. MS parameters selected for analysis of OTPs by QTOF and QqQ MS.

			QTOF conditions		QqQ conditions			
Compound name	Retention time (min)	Ionization mode	Elemental Composition	Theoretical Mass *(m/z)*	Parent *(m/z)*	Product ion (Q)	Product ion (q)	Ion-ratio Q/q
Omeprazole	5.98	ESI+	$C_{17}H_{20}N_3O_3S^+$	346.1225	346.1	136.1	198.1	1.5
OTP 1	1.93	ESI+	$C_9H_{14}NO_2^+$	168.1025	168.1	120.1	150.1	1.9
OTP 2	2.37	ESI+	$C_9H_{12}NO_3^+$	182.0817	182.1	136.1	154.0	13.6
OTP 3	3.86	ESI+	$C_{17}H_{18}N_3O_4^+$	328.1297	328.1	136.1	154.1	9.6
OTP 4	4.09	ESI+	$C_{17}H_{20}N_3O_2^+$	298.1556	298.2	240.1	136.1	1.9
OTP 5	5.68	ESI+	$C_{17}H_{20}N_3O_2S^+$	330.1276	330.2	182.1	150.1	1.2
OTP 6	1.71	ESI+	$C_9H_{12}NO_4^+$	198.0766	198.0	136.1	180.1	7.4
OTP 7-10	3.45 - 3.55 4.82 - 5.03	ESI+	$C_8H_8N_3O_3^+$	194.0566	194.1	194.1	-	-
OTP 11-12	7.21-7.52	ESI+	$C_{17}H_{17}N_3O_3Cl^+$	346.0958	346.1	136.1	154.0	7.1-7.3
OTP 13	7.70	ESI+	$C_{17}H_{18}N_3O_4SCl_2^+$	430.0395	430.1	166.1	150.1	3.8
OTP 14	8.23	ESI+	$C_{17}H_{16}N_3O_3Cl_2^+$	380.0569	380.1	136.1	154.0	7.8
OTP 15-16	8.34-8.46	ESI+	$C_{17}H_{17}N_3O_4SCl_3^+$	464.0005	464.1	184.1	166.1	5.1-5.6
OTP 17	9.15	ESI+	$C_{16}H_{14}N_3O_4SCl_2^+$	414.0082	414.1	286.1	350.1	2.4

LC-MS/MS analysis of surface water and wastewater samples

All the 52 water samples investigated by QTOF MS were re-analyzed by LC-(ESI)-MS/MS QqQ. The LC conditions were the same as used in degradation experiments, in order to obtain comparable retention times. The 17 TPs and parent omeprazole were included in the method (Table 2). For each compound, two SRM transitions were selected based on fragment ions obtained by QTOF MS during degradation experiments. As no fragment ions were observed for OTPs 7-10 in the QTOF mass spectra, a pseudo-SRM transition was recorded involving the protonated molecule as both precursor and product ion. In addition to the acquisition of two transitions and the agreement in retention time, positive findings were confirmed by accomplishment of Q/q ratios [29]. As reference standards of TPs were unavailable, the sample vial with the highest concentration of analyte from degradation experiments was used instead [26].

A summary of the results obtained is shown in Table 3. In agreement with QTOF MS analysis, OTPs 4 and 5 were also detected by QqQ (and their identity confirmed) in several samples of surface water and effluent wastewater. In addition, OTPs 1 and 13 were also detected and identified in some surface water samples (11% of the samples for OTP 1; 11% for OTP 13) and in a notable number of effluent samples (50% of the samples for OTP 13). In terms of signal intensity, OTP 5 was the most abundant compound, as well as the most frequently detected, as it was present in 90% of the effluents and in 26% of the surface waters. In OTP 5 the initial structure of the omeprazole is greatly retained (unlike in some other TPs), supporting that this TP is clearly related with the use of omeprazole. Consequently, OTP 5 is suggested as an appropriate marker compound of the presence of omeprazole in the aquatic environment, and it could be added to targeted methods for detecting pharmaceuticals in environmental water samples.

In order to illustrate the confirmation of positive findings, Figure 6 shows LC-MS/MS chromatograms for OTPs 1, 4, 5 and 13 detected in a surface water sample. Experimental Q/q ratios did not exceed the maximum deviation allowed (±20% [29]), thus confirming the identity of these compounds according to generally accepted criteria.

Table 3. Summary of the results obtained in the monitoring of TPs of omeprazole in IWW, EWW and SW samples using QTOF and QqQ MS analyzers.

	% positive findings					
	QTOF (retrospective)			QqQ		
Compound name	IWW (n=15)	EWW (n=10)	SW (n=27)	IWW (n=15)	EWW (n=10)	SW (n=27)
Omeprazole	-	-	-	-	-	-
OTP 1	-	-	-	-	-	11
OTP 2	-	-	-	-	-	-
OTP 3	-	-	-	-	-	-
OTP 4	-	d (10)	-	-	10	7
OTP 5	-	d (20)	d (7)	-	90	26
OTP 6	-	-	-	-	-	-
OTP 7-10	-	-	-	-	-	-
OTP 11-12	-	-	-	-	-	-
OTP 13	-	-	-	7	50	11
OTP 14	-	-	-	-	-	-
OTP 15-16	-	-	-	-	-	-
OTP 17	-	-	-	-	-	-

d: detected

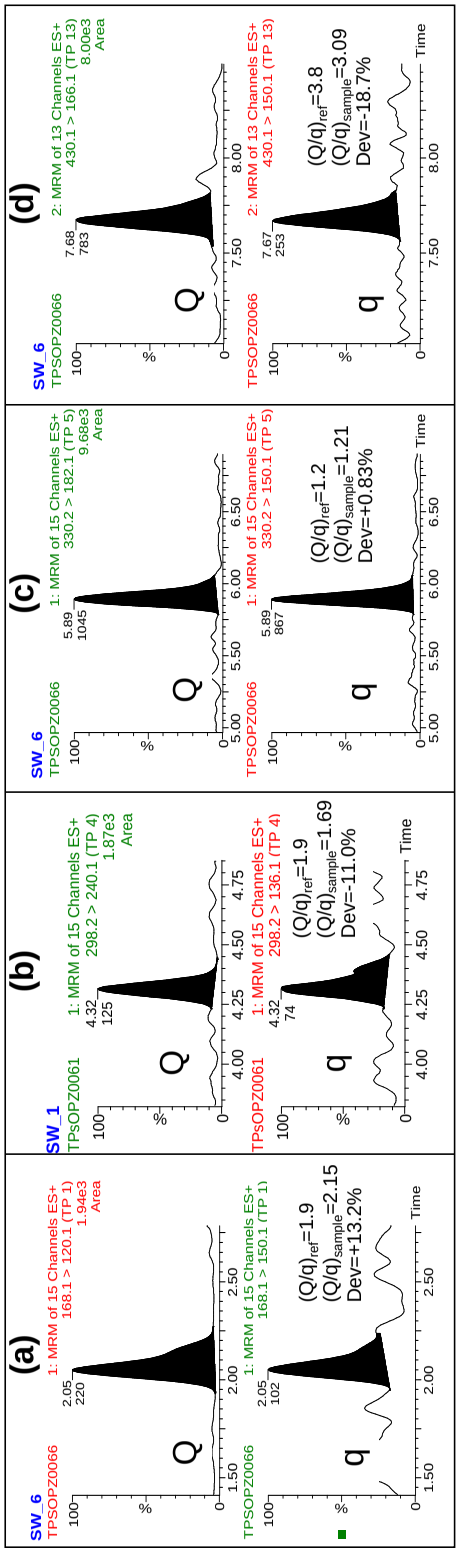

Figure 6. LC-MS/MS QqQ chromatograms for (a) OTP 1, (b) OTP 4, (c) OTP 5 and (d) OTP 13, detected in surface water.

4. CONCLUSIONS

This work reports the omeprazole behavior under hydrolysis, photo-degradation (both sunlight and UV light) and chlorination experiments performed at the laboratory. Altogether, 17 omeprazole TPs were detected and identified by LC-QTOF MS working under MS^E mode. Retrospective LC-QTOF MS analysis of 25 wastewater and 27 surface water samples showed that omeprazole was not present in any of the samples despite its wide consumption. On the contrary, 2 transformation products (OTP 4 and OTP 5) were detected based on the presence of the accurate mass of the protonated molecules. Their identity was subsequently confirmed with independent analysis by LC-MS/MS with triple quadrupole, which better sensitivity made feasible the detection of another two omeprazole TPs (OTP1 and 13). OTP 5 was the most abundant in terms of arbitrary units, and also the most frequently detected, being present in 90% of the effluents and 26% of the surface waters. The elemental composition for the protonated molecule of this TP was found to be $C_{17}H_{20}N_3O_2S^+$ and its tentative chemical structure was suggested as being omeprazole sulfide. Up to our knowledge, this transformation product has not been reported to be present in the aquatic ecosystem in the scientific literature yet. Consequently, it is suggested that future monitoring programs for water analysis should include OTP 5 instead of parent omeprazole to have a more realistic overview on omeprazole impact in the aquatic environment.

ACKNOWLEDGEMENTS

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REFERENCES

[1] M.J. Gómez, O. Malato, I. Ferrer, A. Agüera, A.R. Fernández-Alba. Solid-phase extraction followed by liquid chromatography-time-of-flight- mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study. *J. Environ. Monit.* 2007, *9*, 718.

[2] S. Ortiz de García, G. Pinto Pinto, P. García Encina, R. Irusta Mata. Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. *Sci. Total Environ.* 2013, *444*, 451.

[3] J.P. Besse, J.F. Latour, J. Garric. Anticancer drugs in surface waters. What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? *Environ. Int.* 2012, *39*, 73.

[4] T. Ternes, M. Bonerz, T. Schmidt. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography–electrospray tandem mass spectrometry. *J. Chromatogr. A.* 2001, *938*, 175.

[5] A.L.N. Van Nuijs, I. Tarcomnicu, W. Simons, L. Bervoets, R. Blust, P.G. Jorens, H. Neels, A. Covaci. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater. *Anal. Bioanal. Chem.* 2010, *398*, 2211.

[6] M.A. Sousa, C. Gonçalves, E. Cunha, J. Hajšlová, M.F. Alpendurada. Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS. *Anal. Bioanal. Chem.* 2011, *399*, 807.

[7] M.D. Hernando, M.J. Gómez, A. Agüera, A.R. Fernández-Alba. LC-MS analysis of basic pharmaceuticals (beta-blockers and anti-ulcer agents) in wastewater and surface water. *Trends Anal. Chem.* 2007, *26*, 581.

[8] Y. Valcárcel, S. González Alonso, J.L. Rodríguez-Gil, A. Gil, M. Catalá. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. *Chemosphere.* 2011, *84*, 1336.

[9] E. Gracia-Lor, J.V. Sancho, F. Hernández. Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A.* 2010, *1217*, 622.

[10] R. Rosal, A. Rodríguez, J.A. Perdigón-Melón, A. Petre, E. García-Calvo, M.J. Gómez, A. Agüera, A.R. Fernández-Alba. Occurrence of emerging pollutants in urban

wastewater and their removal through biological treatment followed by ozonation. *Water Res.* 2010, *44*, 578.

[11] E. Zuccato, S. Castiglioni, R. Fanelli. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. *J. Hazard. Mater.* 2005, *122*, 205.

[12] R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández. Building an empirical mass spectra library for screening of organic pollutants by ultra-high-pressure liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 2011, *25*, 355.

[13] F. Hernández, M. Ibáñez, E. Gracia-Lor, J.V. Sancho. Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater. *J. Sep. Sci.* 2011, *34*, 3517.

[14] M. Ibáñez, J.V. Sancho, F. Hernández, D. McMillan, R. Rao. Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-light mass spectrometry. *Trends Anal. Chem.* 2008, *27*, 481.

[15] M. Ibáñez, C. Guerrero, J.V. Sancho, F. Hernández. Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. *J. Chromatogr. A.* 2009, *1216*, 2529.

[16] J.B. Quintana, R. Rodil, R. Cela. Reaction of β-blockers and β-agonist pharmaceuticals with aqueous chlorine. Investigation of kinetics and by-products by liquid chromatography quadrupole time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* 2012, *403*, 2385.

[17] J. Nurmi, J. Pellinen. Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry. *J. Chromatogr. A.* 2011, *1218*, 6712.

[18] M. DellaGreca, M.R. Iesce, L. Previtera, M. Rubino, F. Temussi, M. Brigante. Degradation of lansoprazole and omeprazole in the aquatic environment. *Chemosphere.* 2006, *63*, 1087.

[19] V. Gallardo, M. López-Viota, J. Sierra, M.A. Ruiz. Spectrophotometric and chromatographic determination of omeprazole in pharmaceutical formulations Determination of omeprazole in pharmaceutical formulations V. Gallardo et al. *Pharm. Dev. Technol.* 2009, *14*, 516.

[20] C. Boix, M. Ibáñez, T. Zamora, J.V. Sancho, W.M.A. Niessen, F. Hernández. Identification of new omeprazole metabolites in wastewaters and surface waters, *Sci. Total Environ.* 2013, *706*, 714.

[21] J.L. Acero F.J. Benítez, F.J. Real, M. González. Chlorination of organophosphorus pesticides in natural waters. *J. Hazard. Mater.* 2008, *153*, 320.

[22] A.G. Trovó, R.F.P. Nogueira, A. Agüera, C. Sirtori, A.R. Fernández-Alba. Photodegradation of sulfamethoxazole in various aqueous media: Persistence, toxicity and photoproducts assessment. *Chemosphere.* 2009, *77*, 1292.

[23] L. Bijlsma, C. Boix, W.M.A. Niessen, M. Ibáñez, J.V. Sancho, F. Hernández. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. *Sci. Total Environ.* 2013, *443*, 200.

[24] F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. *Anal. Chim. Acta.* 2011, *684*, 87.

[25] F. Hernández, S. Grimalt, Ó.J. Pozo, J.V. Sancho. Use of ultra-high-pressure liquid chromatography-quadrupole time-of-flight MS to discover the presence of pesticide metabolites in food samples. *J. Sep. Sci.* 2009, *32*, 2245.

[26] F. Hernández, M. Ibáñez, Ó.J. Pozo, J.V. Sancho. Investigating the presence of pesticide transformation products in water by using liquid chromatography-mass spectrometry with different mass analyzers. *J. Mass Spectrom.* 2008, *43*, 173.

[27] S. Grimalt, Ó.J. Pozo, J.V. Sancho, F. Hernández. Use of liquid chromatography coupled to quadrupole time-of-flight mass spectrometry to investigate pesticide residues in fruits. *Anal. Chem.* 2007, *79*, 2833.

[28] M.K.K. Nielsen, S.S. Johansen, P.W. Dalsgaard, K. Linnet. Simultaneous screening and quantification of 52 common pharmaceuticals and drugs of abuse in hair using UPLC-TOF-MS. *Forensic Sci. Int.* 2010, *196*, 85.

[29] European Union Decision 2002/657/EC Off. J. Eur. Commun., L221 pp. 8-36 (12 August 2002).

[30] M. Antonelli, V. Mezzanotte, C. Nurizzo. Wastewater disinfection by UV irradiation: Short and long-term efficiency. *Environ. Eng. Sci.* 2008, *25*, 363.

SUPPLEMENTARY INFORMATION

Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry: degradation experiments

1. Reagents and chemicals

Omeprazole was purchased from Sigma–Aldrich (Madrid, Spain). Standard stock solution was prepared at 100 mg L^{-1} in methanol. An intermediate solution was obtained by ten-fold dilution of the stock solution with methanol. Finally, the standard working solution was prepared by diluting the intermediate solution with Milli Q water.

HPLC-grade methanol, acetonitrile, sodium hydroxide (99%) and formic acid (98-100%) were acquired from Scharlau (Barcelona. Spain). A Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA) was used to obtain the HPLC-grade water. Leucine enkephalin, used as lockmass, was purchased from Sigma-Aldrich.

2. Instrumentation

2.1 LC-ESI-QTOF MS

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 chromatographic separation was performed using an Acquity UPLC BEH C18 1.7 µm, 100 mm × 2.1 mm analytical column (Waters) at a flow rate of 300 µL min^{-1}. The mobile phases used were H_2O and MeOH, both 0.01% HCOOH. The percentage of organic modifier was changed linearly as follows: 0 min, 10%; 9 min, 90%; 11 min, 90%; 11.10 min, 10%; 14 min, 10%. Nitrogen (from a nitrogen generator) was used as the drying gas and nebulizing gas. The gas flow was set at 600 L h^{-1}. TOF MS resolution was approximately 10.000 at full width half maximum (FWHM) at *m/z* 556. MS data were acquired over an *m/z* range of 50–1000. The microchannel plate (MCP) detector potential was set to 1950 V. The capillary voltage was set to 3.5 kV and -3.0 kV in positive

and negative ion modes, respectively. A cone voltage of 25 V was used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 350 °C and the source temperature to 120 °C. The column temperature was set to 40 °C.

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 eV to 40 eV in order to promote fragmentation. The LE and HE functions settings were for both a scan time of 0.15 s and an inter-scan delay of 0.05 s. This approach enables the simultaneous acquisition of both parent (deprotonated or protonated molecules) and fragment ions in a single injection [1-3]. For MS/MS experiments, a collision energy ramp ranging from 15 eV to 40 eV was also used.

Calibrations were conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.05 M NaOH 5% HCOOH diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 10 μL min^{-1}. For automated accurate mass measurement, the lock-spray probe was used, using as lockmass a solution of leucine enkephalin (2 μg mL^{-1}) in acetonitrile:water (50:50) at 0.1% HCOOH pumped at 30 μL min^{-1} through the lock-spray needle. Cone voltages of 80 and 70 V were selected in positive and negative ion modes, respectively, to obtain adequate signal intensity for this compound (~ 400 counts). The protonated (*m/z* 556.2771) or deprotonated (*m/z* 554.2615) molecule of leucine enkephalin was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time.

2.2 LC-ESI-QqQ MS

A Waters Acquity UPLC system was interfaced to a triple quadrupole mass spectrometer (TQD, Waters) with an orthogonal Z-spray-electrospray interface. 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^{-1} and 1200 L h^{-1}, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a pressure of $2 \cdot 10^{-3}$ mbar in the T-Wave collision cell. A capillary voltage of 3.5 kV in positive ionization mode was applied. The interface

temperature was set to 500 ºC and the source temperature to 120 ºC. A dwell time of 0.015 s was selected. Cone and collision energies could not be optimized as reference standards were not available. Instead, typical values of 30 V and 25 eV, respectively, were selected. Seventeen TPs as well as the omeprazole itself were monitored, acquiring two transitions per compound based on the fragment ions observed in the TPs identified along degradation experiments. The same UHPLC conditions indicated in LC-QTOF MS analysis were applied.

Table 1SI. Characteristics of the surface water used in the degradation experiments.

Characteristics	Values
Temperature (ºC, *in situ*)	15.1
pH (*in situ*)	8.5
Conductivity (µS/cm, *in situ*)	810.7
Hardness (expressed as ppm $CaCO_3$)	316.8
Alkalinity (expressed as ppm HCO_3^-)	131.6
Chlorides (expressed as ppm Cl^-)	44.2
Nitrates (expressed as ppm NO_3^-)	3.8
Phosphate (expressed as ppm PO_4^{3-})	2.8
Organic matter (expressed as ppm O_2)	2.2
Sulphates (expressed as ppm SO_4^{2-})	218.7

REFERENCES

[1] R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández. Building an empirical mass spectra library for screening of organic pollutants by ultra-high-pressure liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 2011, *25*, 355.

[2] M. Ibáñez, C. Guerrero, J.V. Sancho, F. Hernández. Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. *J. Chromatogr. A.* 2009, *1216*, 2529.

[3] R.S. Plumb, K.A. Johnson, P. Rainville, B.W. Smith, I.D. Wilson, J.M. Castro-Perez, J.K. Nicholson. UPLC/MSE; a new approach for generating molecular fragment information for biomarker structure elucidation. *Rapid Commun. Mass Spectrom.* 2006, *20*, 1989.

3.3 Artículo Científico 3

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Identification of new omeprazole metabolites in wastewaters and surface waters

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HIGHLIGHTS

- 24 metabolites were identified in urine samples by LC-QTOF MS.
- Parent omeprazole was present at very low concentrations in urine samples.
- The most relevant metabolite presented the same omeprazole exact mass and shared a major fragment ion.
- Up to 14 metabolites were detected and identified by LC-MS/MS QqQ in environmental waters.
- The most detected metabolites are suggested as target analytes to evaluate environmental impact of omeprazole.

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ABSTRACT

Omeprazole is one of the world-wide most consumed pharmaceuticals for treatment of gastric diseases. As opposed to other frequently used pharmaceuticals, omeprazole is scarcely detected in urban wastewaters and environmental waters. This was corroborated in a previous research, where parent omeprazole was not detected while four transformation products (TPs), mainly resulting from hydrolysis, were found in effluent wastewaters and surface waters. However, the low abundance of omeprazole TPs in the water samples together with the fact that omeprazole suffers an extensive metabolism, with a wide range of excretion rates (between 0.01 and 30%), suggests that human urinary metabolites should be investigated in the water environment. In this work, the results obtained in excretion tests after administration of a 40 mg omeprazole dose in three healthy volunteers are reported. Analysis by liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF MS) reported low concentrations of omeprazole in urine. Up to twenty-four omeprazole metabolites (OMs) were detected and tentatively elucidated. The most relevant OM was an omeprazole isomer, which obviously presented the same exact mass (m/z 346.1225), but also shared a major common fragment at m/z 198.0589. Subsequent analyses of surface water and effluent wastewater samples by both LC-QTOF MS and LC-MS/MS with triple quadrupole revealed that this metabolite (named as OM10) was the compound most frequently detected in water samples, followed by OM14a and OM14b. Up to our knowledge, OM10 had not been used before as urinary biomarker of omeprazole in waters. On the contrary, parent omeprazole was never detected in any of the water samples. After this research, it seems clear that monitoring the presence of omeprazole in the aquatic environment should be focused on the OMs suggested in this article instead of the parent compound.

1. Introduction

Environmental contamination by pharmaceuticals (both human and veterinary medicines) is an issue of general concern. They are emerging pollutants widely distributed in the environment, which can enter through different routes (Zuccato et al., 2005). Once a pharmaceutical is administered, it can be excreted unchanged or as metabolites in the urine or feces, reaching the aquatic environment commonly throughout sewage waters (Besse et al., 2012; González Alonso et al., 2010; Ortiz de García et al., 2013).

Omeprazole is one of the most frequently prescribed and administered pharmaceuticals in humans for proton pump inhibition (Andersson et al., 1993; Bruni and Ferreira., 2008; José Gómez et al., 2007; Ortiz de García et al., 2013). As an example, 51,874,630 packages, under prescription, were dispensed in Spain in 2010 (http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf). It is known to act by irreversibly

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IDENTIFICATION OF NEW OMEPRAZOLE METABOLITES IN WASTEWATERS AND SURFACE WATERS

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ABSTRACT

Omeprazole is one of the world-wide most consumed pharmaceuticals for treatment of gastric diseases. As opposed to other frequently used pharmaceuticals, omeprazole is scarcely detected in urban wastewaters and environmental waters. This was corroborated in a previous research, where parent omeprazole was not detected while four transformation products (TPs), mainly resulting from hydrolysis, were found in effluent wastewaters and surface waters. However, the low abundance of omeprazole TPs in the water samples together with the fact that omeprazole suffers an extensive metabolism, with a wide range of excretion rates (between 0.01 and 30%), suggests that human urinary metabolites should be investigated in the water environment. In this work, the results obtained in excretion tests after administration of a 40 mg omeprazole dose in three healthy volunteers are reported. Analysis by liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF MS) reported low concentrations of omeprazole in urine. Up to twenty-four omeprazole metabolites (OMs) were detected and tentatively elucidated. The most relevant OM was an omeprazole isomer, which obviously presented the same exact mass (*m*/*z* 346.1225), but also shared a major common fragment at *m*/*z* 198.0589. Subsequent analyses of surface water and effluent wastewater samples by both LC-QTOF MS and LC-MS/MS with triple quadrupole revealed that this metabolite (named as OM10) was the compound most frequently detected in water samples, followed by OM14a and OM14b. Up to our knowledge, OM10

had not been used before as urinary biomarker of omeprazole in waters. On the contrary, parent omeprazole was never detected in any of the water samples. After this research, it seems clear that monitoring the presence of omeprazole in the aquatic environment should be focused on the OMs suggested in this article instead of the parent compound.

Keywords

Omeprazole, metabolites, urine, time-of-flight mass spectrometry, triple quadrupole mass spectrometry, water samples.

Highlights

- 24 metabolites were identified in urine samples by LC-QTOF MS.
- Parent omeprazole was present at very low concentrations in urine samples.
- The most relevant metabolite presented the same omeprazole exact mass and shared a major fragment ion.
- Up to 14 metabolites were detected and identified by LC-MS/MS QqQ in environmental waters.
- The most detected metabolites are suggested as target analytes to evaluate environmental impact of omeprazole.

1. INTRODUCTION

Environmental contamination by pharmaceuticals (both human and veterinary medicines) is an issue of general concern. They are emerging pollutants widely distributed in the environment, which can enter through different routes (Zuccato et al., 2005). Once a pharmaceutical is administered, it can be excreted unchanged or as metabolites in the urine or feces, reaching the aquatic environment commonly throughout sewage waters (Besse et al., 2012, González Alonso et al., 2010 and Ortiz de García et al., 2013).

Omeprazole is one of the most frequently prescribed and administered pharmaceuticals in humans for proton pump inhibition (Andersson et al., 1993, Bruni and Ferreira, 2008, José Gómez et al., 2007 and Ortiz de García et al., 2013). As an example, 51,874,630 packages, under prescription, were dispensed in Spain in 2010 (http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf). It is known to act by irreversibly blocking the terminal stage of gastric acid secretion in the gut. This compound is reported to be metabolized by the enzyme CYP2C19 to form the 5-hydroxy metabolite whereas CYP3A4 catalyzes the sulfone formation (Kanazawa et al., 2002 and Rost et al., 1995). About 80% of orally administered omeprazole dose is excreted in urine as metabolites, whereas the remainder is excreted in the feces, mainly from biliary secretion (Andersson et al., 1993). Different percentages of omeprazole excretion (as intact parent) can be found in the literature, ranging from 0.01% (Besse et al., 2008) to 5% (Hernando et al., 2007), or even up to 30% (Ortiz de García et al., 2013). This variation has been justified based on the different enzymatic activity of each individual. Sulfonated and 5-hydroxylated compound are the major omeprazole metabolites (OMs) found in plasma (Espinosa Bosch et al., 2007 and Song and Naidong, 2006), whereas in urine the 5-hydroxylated OM is the predominant one (Petsalo et al., 2008) (Fig. 1). The concentration of omeprazole sulfide, another OM reported in the literature, is usually too low to be determined in plasma, and it is also negligible in urine (Rezk et al., 2006).

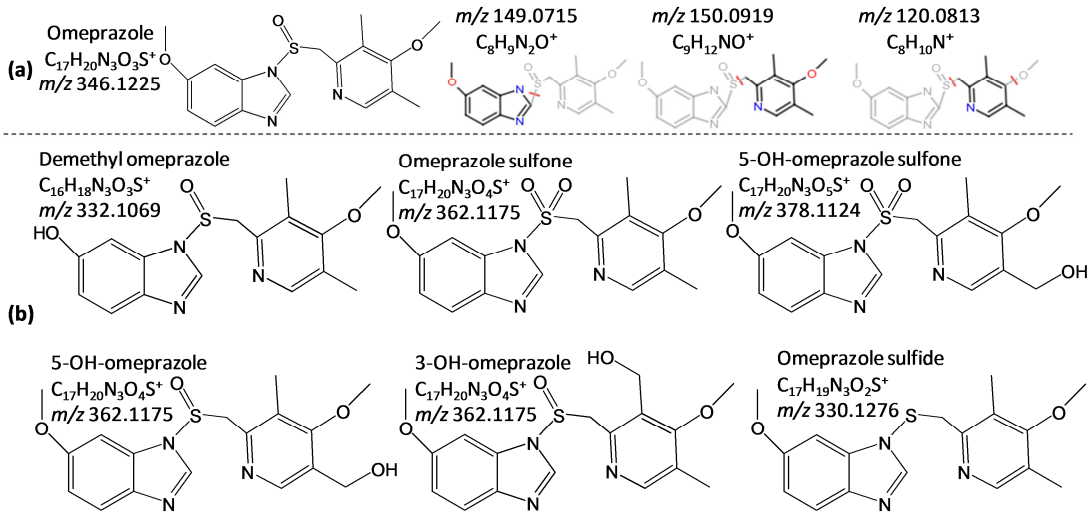

Fig. 1. (a) Structure of omeprazole and some important fragment ions (b) Omeprazole metabolites reported in the literature.

Several analytical methods have been reported for determination of omeprazole in plasma (Kanazawa et al., 2002, Macek et al., 2007, Rost et al., 1995 and Song and Naidong, 2006) while only a few articles deal with analysis of urine samples. Petsalo et al. (2008) focused on the determination of the 3-hydroxy-, 5-hydroxy-, demethyl-, and sulfone-OMs and omeprazole itself in urine. It was not possible to detect 3-hydroxy OM, and the concentrations of omeprazole and its sulfone OM were very low. Chung et al. (2004) reported the detection of four unconjugated and two conjugated OMs in horse urine by LC-MS.

The available literature on omeprazole determination highlights the application of liquid chromatography (LC) as the most appropriate analytical tool for this compound (Kanazawa et al., 2002, Petsalo et al., 2008, Song and Naidong, 2006 and Ternes et al., 2001). Although some methods have made use of UV as detection technique (Rezk et al., 2006), currently mass spectrometry (MS) is the technique of choice for determination of omeprazole, particularly LC coupled to tandem MS (MS/MS), the advantages of which, short analytical run time as well as excellent selectivity and sensitivity, are widely recognized (Espinosa Bosch et al., 2007). While LC-MS/MS with triple quadrupole (QqQ) analyzer is the workhorse for quantitative analysis of pharmaceuticals, omeprazole

included, in the aquatic environment (Castiglioni et al., 2004, Gracia-Lor et al., 2010, Van Nuijs et al., 2010 and Zuccato et al., 2005), LC coupled to high resolution mass spectrometry (HRMS) such as Orbitrap (Calza et al., 2012 and Thevis et al., 2011), FTMS (Awasthi et al., 2012) or time-of-flight MS (Ibáñez et al., 2004 and Ibáñez et al., 2006) is a powerful analytical tool for investigation of metabolites and/or transformation products (TPs) in water. These HR MS techniques are also appropriate to perform metabolism studies of pharmaceuticals within the biomedical field (Corcoran et al., 2000 and Hopfgartner et al., 1999) due to the accurate-mass full-spectrum acquisitions provided by these analyzers.

Considering the high consumption of omeprazole and the reported excretion rates of up to 20% as intact omeprazole, one might expect to find this compound in urban wastewater, or even in environmental waters. Nevertheless, its detection in water samples is rarely reported. Additionally, in our previous study on omeprazole degradation (Boix et al., 2013), only four low-abundant TPs were rarely found in water samples, with omeprazole sulfide the most frequently detected. The initial hypothesis on a possible degradation of omeprazole in waters was thus discarded and a detailed study on human urinary metabolites of omeprazole was initiated. This paper pursues the detection and elucidation of urinary OMs making use of LC-QTOF MS. Subsequently, 27 surface water (SW) and 25 wastewater (WW) samples have been analyzed by LC-QTOF MS and LC-MS/MS QqQ to investigate the presence of OMs.

2. EXPERIMENTAL

2.1. Reagents and chemicals

See Supplementary Information (SI).

2.2. Instrumentation

2.2.1. UHPLC-QTOF MS

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 electrospray ionization (ESI) interface operating in positive and negative ion modes (For further details, see SI).

QTOF data were acquired under MS^E mode, an approach that enables the simultaneous acquisition of both parent protonated molecules and fragment ions in a single injection. So, two acquisition functions with different collision energies were created. The first one, the low energy (LE) function, selecting a collision energy of 4 eV, and the second one, the high energy (HE) function, with a collision energy ramp ranging from 15 eV to 40 eV (Díaz et al., 2011, Hernández et al., 2011a, Hernández et al., 2011b and Plumb et al., 2006).

2.2.2. UHPLC-MS/MS QqQ

A Waters Acquity UPLC system was interfaced to a triple quadrupole mass spectrometer (TQD, Waters) with an orthogonal Z-spray–ESI interface. Capillary voltages of 3.5 and – 3.0 kV were used in positive and negative ionization mode, respectively (For further details, see SI).

2.3. Analysis of metabolites excreted in urine

Urine samples were collected from three healthy volunteers with different gender (1 male, 2 females) and origins (2 Europeans and 1 Latin–American, all living in Europe). Each volunteer ingested an oral dose of 40 mg omeprazole (i.e., two 20 mg capsules). Urine samples were collected before ingestion of the drug (control sample), and after 15 min, 1, 3.5, 6.5, 9, 12 and 24 h of drug administration. Polyethylene bottles were used for

collecting samples, which were immediately stored at – 18 °C until analysis. The study protocol was approved by an ethical committee (University Jaume I, Spain).

For analysis of urine, 1 mL of sample previously centrifuged (10,000 r.p.m., 10 min), was two-fold diluted with Milli-Q water. After that, 50 µL was directly injected in the UHPLC-QTOF MS system.

For the determination of metabolites released from glucuronide conjugates, 1 mL of centrifuged urine was buffered with 50 µL acetic acid/ammonium acetate (pH 5.5). After being hydrolyzed overnight with 200 units of β-D-glucuronidase at 37 °C, 50 µL of the hydrolyzed mixture was injected in the UHPLC-QTOF MS system (Hernández et al., 2004).

2.4. Retrospective QTOF MS analysis of water samples

25 wastewater samples (15 influents and 10 effluents) were collected from three different wastewater treatment plants (WWTPs) of the Valencian region (Eastern Spain), whose anonymity must be respected, from June 2008 to December 2010. 27 surface waters were also sampled from several points located in the same area in October 2010. All these samples had been used previously in different studies performed at our lab using UHPLC-QTOF MS for their analysis.

2.5. Data processing

QTOF MS data were processed using MetaboLynx XS and ChromaLynx XS application managers (Waters Micromass vs 4.1). Regarding data from triple quadrupole, TargetLynx software (also from Waters) was used (More details in ref. (Boix et al., 2013)).

3. RESULTS AND DISCUSSION

3.1. Urinary excretion of parent omeprazole

Omeprazole was detected at low concentration levels (not quantified) in non-hydrolyzed urine from the two European volunteers approximately between 1 and 3 h after administration of the drug. In the third volunteer (Latin–American female), the drug was not detected in any of the urine samples collected. This would be in accordance with the scientific literature where different levels of excretion for omeprazole have been reported (Besse et al., 2008, Hernando et al., 2007, Ortiz de García et al., 2013 and Zuccato et al., 2005).

Interestingly, the narrow window eXtracted Ion Chromatogram (nw-XIC, 0.02 Da) at the exact mass of omeprazole ($[M + H]^+$ m/z 346.1225), showed a highly abundant peak at different retention time (5.88 min for omeprazole, 4.91 min for this compound, OM11a) (Fig. S1a). Although HE MS spectra of both compounds were quite different (e.g. specific fragment ions: m/z 136 and 151 for omeprazole; m/z 138 and 149 for OM11a, Figs. S1b,c), they shared an important common fragment at m/z 198.0589. This fact cannot be neglected, as it might lead to a false positive of omeprazole even by LC-MS/MS if only the transition 346.1 > 198.1 (the most commonly reported in the literature) is acquired (Petsalo et al., 2008, Macek et al., 2007 and Song and Naidong, 2006), without sufficient chromatographic separation. This is of relevance taking into account the high abundance of this metabolite.

3.2. Elucidation of metabolites

Table 1 shows the elemental composition, mass errors of the (de)protonated molecule and fragments ions, and retention time for omeprazole and twenty-four OMs detected in hydrolyzed and non-hydrolyzed urine. Fig. 1 shows the structures suggested for the main fragment ions together with the omeprazole metabolites reported in the literature. Tentative structure proposals for OMs identified in this work are given in Fig. 2. All metabolites were detected in positive-ion mode except for the OM12, which was only detected in negative-ion mode.

Table 1. Elemental composition, retention time and mass errors (in mDa) of (de)protonated molecule and fragment ions proposed for omeprazole metabolites.

Proposed compound/ Ret.Time (min)	Elemental Composition	Exact mass (*m/z*)	Mass Error (mDa)	Proposed compound/ Ret. Time (min)	Elemental Composition	Exact mass (*m/z*)	Mass Error (mDa)	Proposed compound/ RetTime (min)	Elemental Composition	Exact mass (*m/z*)	Mass Error (mDa)
Omeprazole	$C_{17}H_{20}N_3O_3S^+$	346.1225	1.4	OM6	$C_{17}H_{18}N_3O_4^+$	328.1297	0.5	OM9	$C_{17}H_{20}N_3O_6S^+$	394.1073	0.8
5.98	$C_9H_{12}NO_2S^+$	198.0589	0.8	4.2	$C_9H_{11}N_2O_3^+$	195.0707	2.6	4.48	$C_{17}H_{18}N_3O_5S^+$	376.0967	1.9
	$C_9H_{10}NOS^+$	180.0483	0.8		$C_8H_{10}N^+$	120.0813	0.5		$C_{17}H_{18}N_3O_3^+$	312.1342	1.2
	$C_9H_{13}NO^{+\bullet}$	151.0997	0.9						$C_{16}H_{14}N_3O_3^+$	296.1035	1.1
	$C_9H_{12}NO^+$	150.0919	0.2	OM7a	$C_{17}H_{20}N_3O_5S^+$	378.1124	0.7		$C_8H_7N_2O_2S^+$	195.0228	2.4
	$C_8H_9N_2O^+$	149.0715	0.4	4.43	$C_9H_{12}NO_4S^+$	230.0487	0.7		$C_8H_9N_2O^+$	149.0715	0.7
	$C_8H_{10}NO^+$	136.0762	1.3		$C_9H_{10}NO_3S^+$	212.0381	1.4				
	$C_8H_{11}N^{+\bullet}$	121.0891	0.8		$C_8H_{10}NO_3^+$	168.0661	1.1	OM10	$C_{17}H_{18}N_3O_4S^+$	360.1018	0.1
	$C_8H_{10}N^+$	120.0813	1.5		$C_8H_9N_2O^+$	149.0715	1.3	4.78	$C_{17}H_{17}N_3O_4^{+\bullet}$	327.1219	4.2
OM1	$C_{20}H_{25}N_4O_5S_2^+$	465.1266	0.0	[b]OM7b	$C_{17}H_{20}N_3O_5S^+$	378.1124	1.5		$C_{16}H_{14}N_3O_4^+$	312.0984	0.3
3.13	$C_{17}H_{18}N_3O_3S^+$	344.1069	0.8	4.9	$C_9H_{12}NO_4S^+$	230.0487	0.7		$C_9H_{10}NO_3S^+$	212.0381	0.6
Phase II	$C_{17}H_{19}N_3O_3^{+\bullet}$	313.1426	1.2		$C_9H_{12}NO_2^+$	166.0868	1.4		$C_9H_{10}NO_3^+$	180.0661	0.7
Cys-conj	$C_{16}H_{16}N_3O_3^+$	298.1192	0.6		$C_8H_9N_2O^+$	149.0715	1.8		$C_8H_9N_2O^+$	149.0715	0.7
	$C_{16}H_{18}N_3O_2^+$	284.1399	1.0		$C_8H_{12}NO^+$	138.0919	1.3				
	$C_{15}H_{14}N_3O^+$	252.1137	0.7	OM7c	$C_{17}H_{20}N_3O_5S^+$	378.1124	1.2	[b]OM11a	$C_{17}H_{20}N_3O_3S^+$	346.1225	2.3
				5.23	$C_{17}H_{20}N_3O_3^+$	314.1505	2.8	4.90	$C_{17}H_{19}N_3O_3^{+\bullet}$	313.1426	4.8
OM2a	$C_{23}H_{28}N_3O_9S^+$	522.1546	0.8		$C_9H_{12}NO_4S^+$	230.0487	1.9		$C_{16}H_{16}N_3O_3^+$	298.1192	1.2
3.59	$C_{17}H_{20}N_3O_3S^+$	346.1225	0.6		$C_9H_{11}N_2OS^+$	195.0228	1.4		$C_9H_{12}NO_2S^+$	198.0589	0.9
Phase II	$C_{17}H_{19}N_3O_3^{+\bullet}$	313.1426	1.9		$C_9H_{10}NO_3^+$	180.0661	1.1		$C_9H_{12}NO_2^+$	166.0868	0.4
Gluc-conj	$C_{16}H_{16}N_3O_3^+$	298.1192	1.4		$C_9H_{12}NO_2^+$	166.0868	1.0		$C_8H_{10}NO_2^+$	152.0712	1.3
	$C_9H_{12}NOS^+$	182.0640	0.9		$C_8H_9N_2O^+$	149.0715	1.8		$C_8H_9N_2O^+$	149.0715	0.5
	$C_8H_9N_2O_2^+$	165.0664	1.2		$C_8H_{12}NO^+$	138.0919	1.6		$C_8H_{12}NO^+$	138.0919	0.9
	$C_8H_{10}N^+$	120.0813	1.8	OM7d	$C_{17}H_{20}N_3O_5S^+$	378.1124	0.4	[a]OM11b	$C_{17}H_{20}N_3O_3S^+$	346.1225	1.5
OM2b	$C_{23}H_{28}N_3O_9S^+$	522.1546	0.7	5.32	$C_{17}H_{18}N_3O_4S^+$	360.1018	1.6	4.52	$C_{17}H_{19}N_3O_3^{+\bullet}$	313.1426	0.6
4.48	$C_{23}H_{28}N_3O_9^+$	489.1747	0.8		$C_{17}H_{18}N_3O_2^+$	296.1399	1.9		$C_{16}H_{16}N_3O_3^+$	298.1192	0.8
Phase II	$C_{17}H_{20}N_3O_3S^+$	346.1225	1.6		$C_{16}H_{14}N_3O_2^+$	280.1086	0.3		$C_9H_{12}NO_2S^+$	198.0589	1.3
Gluc-conj	$C_{17}H_{18}N_3O_2S^+$	328.1120	1.6		$C_9H_{12}NO_4S^+$	230.0487	0.5		$C_9H_{12}NOS^+$	182.0640	0.1
	$C_{17}H_{19}N_3O_3^{+\bullet}$	313.1426	0.2		$C_{15}H_{12}N_3O_2$	266.0930	1.4		$C_8H_9N_2O_2^+$	165.0664	1.2
	$C_{16}H_{16}N_3O_3^+$	298.1192	0.3		$C_9H_{12}NO_2^+$	166.0868	1.0		$C_9H_{12}NO^+$	150.0919	1.1
	$C_9H_{12}NO_2S^+$	198.0589	0.1		$C_8H_9N_2O^+$	149.0715	1.1		$C_8H_{10}N^+$	120.0813	1.5
	$C_9H_{12}NO_2^+$	166.0868	1.4		$C_8H_{12}NO^+$	138.0919	1.8				
	$C_8H_9N_2O^+$	149.0715	0.5	OM7e	$C_{17}H_{20}N_3O_5S^+$	378.1124	0.9	OM12	$C_{17}H_{18}N_3O_7S_2^-$	440.0586	0.6

Table 1. Elemental composition, retention time and mass errors (in mDa) of (de)protonated molecule and fragment ions proposed for omeprazole metabolites (Cont.).

Proposed compound/ Ret.Time (min)	Elemental Composition	Exact mass (*m/z*)	Mass Error (mDa)
OM2c	$C_{23}H_{28}N_3O_9S^+$	522.1546	0.9
4.82	$C_{17}H_{20}N_3O_3S^+$	346.1225	0.7
Phase II	$C_{17}H_{18}N_3O_2S^+$	328.1120	1.1
Gluc-conj	$C_{17}H_{19}N_3O_3^{+\bullet}$	313.1426	2.6
	$C_{16}H_{16}N_3O_3^+$	298.1192	1.8
	$C_9H_{12}NO_2S^+$	198.0589	0.9
	$C_9H_{10}NO_3^+$	180.0661	0.7
	$C_9H_{10}NO_2^+$	164.0712	0.7
	$C_8H_9N_2O^+$	149.0715	0.5
	$C_8H_{12}NO^+$	138.0919	0.9
OM3	$C_{16}H_{18}N_3O_4S^+$	348.1018	0.7
3.60	$C_9H_{12}NO_3S^+$	214.0538	0.9
	$C_9H_{10}NO_2S^+$	196.0432	0.5
	$C_8H_{10}NO_2^+$	152.0712	1.3
	$C_7H_7N_2O^+$	135.0558	1.4
OM4	$C_{16}H_{18}N_3O_3S^+$	332.1069	0.7
3.73	$C_{16}H_{17}N_3O_3^{+\bullet}$	299.1270	2.9
	$C_{15}H_{16}N_3O_3^+$	284.1035	1.6
	$C_9H_{12}NO_2S^+$	198.0589	0.3
	$C_8H_{10}NO_2^+$	152.0712	1.1
	$C_7H_7N_2O^+$	135.0558	1.8
OM5	$C_{16}H_{18}N_3O_5S_2^+$	396.0688	0.4
3.94	$C_{16}H_{18}N_3O_5S^+$	364.0967	1.2
Sulf-conj	$C_{16}H_{18}N_3O_2S^+$	316.1120	0.2
	$C_{16}H_{17}N_3O_2^{+\bullet}$	283.1321	2.3
	$C_{15}H_{14}N_3O_2^+$	268.1086	1.4
	$C_9H_{12}NOS^+$	182.0640	0.1
	$C_9H_{12}NO^+$	150.0919	0.4
	$C_7H_7N_2O^+$	135.0558	1.4
	$C_8H_{10}N^+$	120.0813	0.9
5.55	$C_{17}H_{18}N_3O_4S^+$	360.1018	0.1
	$C_9H_{12}NO_4S^+$	230.0487	0.7
	$C_9H_{12}NO_2^+$	166.0868	1.0
	$C_8H_9N_2O^+$	149.0715	1.6
OM8a	$C_{17}H_{20}N_3O_4S^+$	362.1175	1.5
4.50	$C_{17}H_{18}N_3O_3S^+$	344.1069	0.9
	$C_{17}H_{19}N_3O_4^{+\bullet}$	329.1376	1.6
	$C_{16}H_{16}N_3O_4^+$	314.1141	1.0
	$C_{17}H_{16}N_3O_3^+$	310.1192	1.2
	$C_9H_{10}NO_3S^+$	212.0381	1.1
	$C_9H_{12}NO_2S^+$	198.0589	0.5
	$C_8H_{10}NO_2^+$	152.0712	1.6
	$C_9H_{12}NO^+$	150.0919	1.7
	$C_8H_9N_2O^+$	149.0715	1.5
OM8b	$C_{17}H_{20}N_3O_4S^+$	362.1175	1.5
5.06	$C_{17}H_{18}N_3O_3S^+$	344.1069	1.9
	$C_{17}H_{19}N_3O_4^{+\bullet}$	329.1376	1.8
	$C_9H_{12}NO_3S^+$	214.0538	0.9
	$C_9H_{10}NO_2S^+$	196.0432	1.7
	$C_9H_{14}NO_3^+$	184.0974	0.4
	$C_9H_{12}NO^+$	150.0919	1.8
	$C_8H_9N_2O^+$	149.0715	0.5
OM8c	$C_{17}H_{20}N_3O_4S^+$	362.1175	1.5
5.55	$C_9H_{12}NO_3S^+$	214.0538	1.8
	$C_9H_{14}NO_3^+$	184.0974	1.1
	$C_8H_7N_2OS^+$	179.0279	0.2
	$C_9H_{12}NO_2^+$	166.0868	1.0
	$C_9H_{12}NO^+$	150.0919	1.1
	$C_8H_9N_2O^+$	149.0715	1.6
	$C_8H_{10}N^+$	120.0813	0.2
4.59	$C_{16}H_{16}N_3O_6S_2^-$	410.0481	1.1
ESI-	$C_{16}H_{14}N_3O_2S^-$	312.0807	1.4
Sulf-conj	O_3S^-	79.9568	1.4
OM13	$C_{16}H_{16}N_3O_4S^+$	346.0862	1.3
3.35	$C_{16}H_{15}N_3O_4^{+\bullet}$	313.1063	1.4
	$C_{16}H_{16}N_3O_3^+$	298.1192	1.6
	$C_9H_{10}NO_3S^+$	212.0381	0.8
	$C_8H_6NO_2S^+$	180.0119	0.9
	$C_8H_8NO_2^+$	150.0555	0.3
	$C_7H_7N_2O^+$	135.0558	1.1
OM14a	$C_{16}H_{18}N_3O_2S^+$	316.1120	1.5
4.52	$C_{16}H_{17}N_3O_2^{+\bullet}$	283.1321	2.3
	$C_{15}H_{14}N_3O_2^+$	268.1089	1.8
	$C_9H_{12}NOS^+$	182.0640	0.1
	$C_9H_{12}NO^+$	150.0919	0.4
	$C_7H_7N_2O^+$	135.0558	1.1
	$C_8H_{10}N^+$	120.0813	1.5
OM14b	$C_{16}H_{18}N_3O_2S^+$	316.1120	1.5
5.01	$C_{16}H_{17}N_3O_2^{+\bullet}$	283.1321	2.3
4-OH	$C_{15}H_{14}N_3O_2^+$	268.1086	1.4
omeprazole	$C_8H_{10}NOS^+$	168.0483	0.4
sulfide	$C_8H_9N_2O^+$	149.0715	1.8
	$C_8H_{11}NO^+$	136.0762	0.7

Cys-conj: cysteine conjugate; Glu-conj: glucuronide conjugate; Sulf-conj: sulfate conjugate

[a] Phase I metabolite that only appeared after hydrolysis

[b] Metabolites phase I which increased after hydrolysis

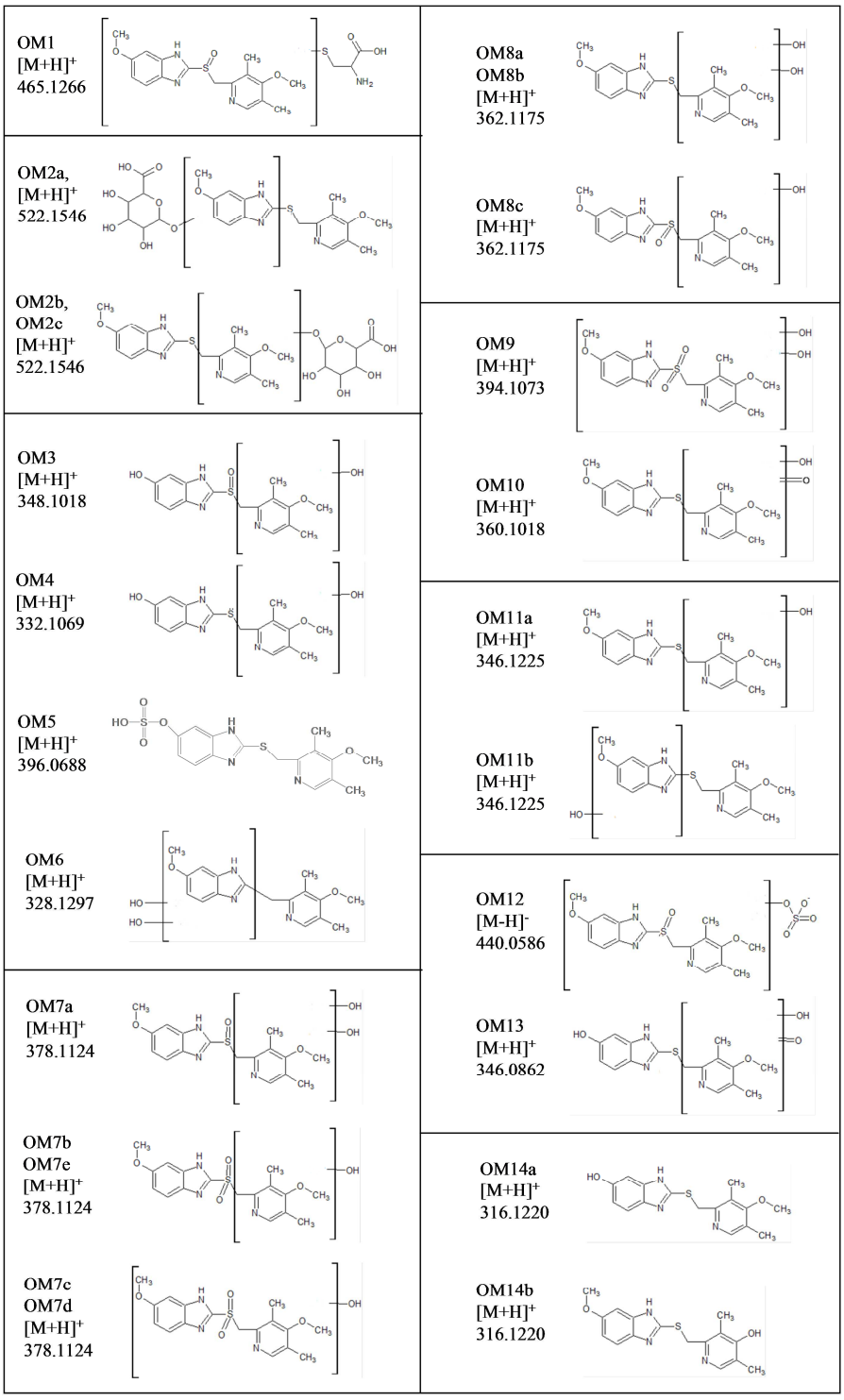

Fig. 2. Suggested structures for urinary OMs detected by UHPLC-QTOF MS after omeprazole oral administration.

Different conjugated metabolites, as cysteine (OM1), glucuronides (OM2a, OM2b, and OM2c) and sulfates (OM5 and OM12) were directly detected (Fig. 2) in the non-hydrolyzed urine. After hydrolysis, a decrease of cysteine and glucuronide conjugates was observed, as well as the corresponding increase in abundance of the OM11a and OM7d. Additionally, a new peak appeared, corresponding to $[C_{17}H_{20}N_3O_3S]^+$ (OM11b) (*m/z* 346.1225, Δ = 1.5 mDa), with the same elemental composition as omeprazole and its isomeric metabolite OM11a, but eluting even earlier (4.52 min). With these data, it seems that OM7d and OM11a are partly conjugated while OM11b is fully conjugated prior to urinary excretion. Based on similarities in their fragmentation, OM11a and OM11b would correspond to the free forms of OM2(b,c) and OM2a, respectively.

The compound OM4 $[C_{16}H_{18}N_3O_3S]^+$, *m/z* 332.1069 (Δ = 0.7 mDa; 3.73 min), has the same exact mass as the reported demethyl-OM (Kanazawa et al., 2002, Petsalo et al., 2008 and Rost et al., 1995). However, OM4 was not considered to match demethyl-OM, as the spectrum of OM4 shows the specific loss of *m/z* 32.9799 ([$^{\bullet}$SH]) related to the reduction of the sulfoxide group.

The loss of the thiol radical [$^{\bullet}$SH] from the protonated molecule was observed in ESI positive for all OMs when the sulfoxide moiety was converted into a sulfide. Initially, cyclation of omeprazole molecule rendering a terminal thiol group was considered, as reported to occur under acidic conditions (Bruni and Ferreira, 2008, DellaGreca et al., 2006 and Weidolf and Castagnoli, 2001). However, in the MS/MS spectrum of the 4-hydroxy omeprazole sulfide reference standard ($C_{16}H_{18}N_3O_2S^+$, *m/z* 316.1120), the loss of the thiol radical [$^{\bullet}$SH] was also observed (Fig. S2a). Therefore, the initial hypothesis of cyclization was rejected. The [$^{\bullet}$SH] loss (32.9799 Da) from the thioether group could be explained from a homolytic cleavage and intermediate binding to the hydrogen at the imidazolyl-N to form [$^{\bullet}$SH] (Fig. S2b). Although the mass error was relatively high (around 4 mDa), other elemental compositions could not be assigned. A subsequent homolytic cleavage, involving the loss of a methyl radical [$^{\bullet}CH_3$] is observed as well (see Fig. S2c). The [$^{\bullet}$SH] loss observed for many OMs suggested the reduction of the sulfoxide group to sulfide.

Regarding the position at which hydroxylation has occurred (for OM4 but also for OM3, OM7abe, OM8abc, OM10 and OM11a), it was justified according to the fragment ions observed in their spectra. So, two fragments ions at *m/z* 149.0715 ($C_8H_9N_2O^+$) and 135.0558 ($C_7H_7N_2O^+$) (see Table 1 and Fig. 1a) correspond to the benzimidazole ring of the original omeprazole molecule and to its demethylated fragment ion, respectively. The presence of one of these fragment ions would therefore imply that hydroxylation has taken place in the other side of the molecule, i.e., in the pyridine ring.

In the case of OM6 (and also OM11b), the hydroxylation was located in the benzimidazole ring based on the presence of the fragment ions at *m/z* 195.0770 ($C_9H_{11}N_2O_3^+$) and 150.0919 ($C_9H_{12}NO^+$), respectively (see Table 1 and Fig. 1a).

Five di-oxygenated OM7s $[C_{17}H_{20}N_3O_5S]^+$ (*m/z* 378.1124) and three mono-oxygenated OM8s $[C_{17}H_{20}N_3O_4S]^+$ (*m/z* 362.1175) were also observed (Fig. 2). However, it was not possible to predict at which position the hydroxylations occurred, as the fragment ions did not provide enough information. The SO_2 loss observed for OM7b–7e indicates the presence of the sulfone group in the molecule. Regarding OM7a, the sulfoxide group was maintained (SO_2 loss not observed) therefore suggesting a double hydroxylation. A previously searched metabolite (omeprazole sulfone-N-oxide, $[C_{17}H_{20}N_3O_5S]^+$) (Hernández et al., 2011b) might also be one of the OM7s. However, no characteristic fragmentation from an N-oxide (involving losses of O, $OH^\bullet$ and H_2O) was observed (Chen et al., 2005).

Other reported OMs, like 5-hydroxy omeprazole (Hernández et al., 2011b, Kanazawa et al., 2002, Petsalo et al., 2008, Rezk et al., 2006, Rost et al., 1995 and Song and Naidong, 2006), 3-hydroxy omeprazole (Kanazawa et al., 2002 and Petsalo et al., 2008) and omeprazole sulfone (Kanazawa et al., 2002, Petsalo et al., 2008, Rezk et al., 2006 and Rost et al., 1995) matched with the exact mass of metabolites 8 a,b,c. Based on the structure proposed for these metabolites according to the observed fragmentation, none of them could be assigned to omeprazole-sulfone (OM8a,b are sulfides and OM8c is a sulfoxide). Moreover, 3- and 5- hydroxy omeprazole could only be related with OM8c. However, after injecting the 5-hydroxy omeprazole reference standard, its retention time and mass spectra were

not in agreement with OM8c. Therefore, omeprazole-sulfone and 5-hydroxy omeprazole were discarded as candidates for the group of OM8 metabolites, while 3-hydroxy omeprazole could still be a plausible candidate for OM8c.

It is worth to mention the detection of OM13 $[C_{16}H_{16}N_3O_4S]^+$ (*m*/*z* 346.0862, ΔmDa = 1.3). This compound shared the same nominal mass than omeprazole (346) and its isomers OM11a and OM11b, but it had different exact mass and retention time. The potential of HRMS allowed differentiating these compounds with different elemental compositions. In this case, the elemental composition for OM13 suggested a demethylation (fragment ion at *m*/*z* 135.0558), sulfoxide-reduction (loss of *m*/*z* 32.9799, [$^{\bullet}$SH]) and subsequent hydroxylation and oxidation (fragment ions at *m*/*z* 212.0381 and 150.0555) from parent omeprazole (Table 1, Fig. 2).

Two isomers, OM14a and OM14b ($[C_{16}H_{18}N_3O_2S]^+$, *m*/*z* 316.1220), eluting at 4.52 and 5.01 min, respectively, are the result of sulfoxide reduction (loss of *m*/*z* 32.9799, [$^{\bullet}$SH]) and demethylation reactions in the omeprazole structure. OM14b presented two fragment ions at *m*/*z* 136.0762 ($C_8H_{11}NO^+$) and 149.0715 ($C_8H_9N_2O^+$) showing that demethylathion occurred in the pyridine ring. The metabolite OM14b corresponds to the already reported 4-hydroxy omeprazole sulfide (Hernández et al., 2011b), and the identity was confirmed with the reference standard available at our laboratory. Regarding the metabolite OM14a, the fragment ions at *m*/*z* 135.0558 ($C_7H_7N_2O^+$) and 150.0919 ($C_9H_{12}NO^+$) indicated that the compound was demethylated at the benzimidazole group.

As an illustrative example, the elucidation process for the two most abundant metabolites, OM10 and OM11a, is discussed in detail in SI.

Up to our knowledge, some of the metabolites reported in this paper have never been investigated in water samples. Reference standards are not commercially available for most of these compounds and therefore their identity, although strongly supported by our QTOF MS accurate-mass data, could not be unequivocally confirmed.

3.3. Retrospective analysis of water samples by UHPLC-QTOF MS

After the study performed on urinary metabolites of omeprazole, the presence of the 24 identified metabolites was investigated in water samples (Table S1). To this aim, a total of 52 samples (15 influent wastewater (IWW), 10 effluent wastewater (EWW) and 27 surface water (SW)) previously analyzed by UHPLC-QTOF MS were retrospectively re-examined, this is, without the need of additional sample injections.

Retrospective analysis was made by performing eXtract Ion Chromatograms using narrow mass windows (± 10 mDa) at the metabolites exact *m/z*-values (nw-XICs). Confirmation of the identity of the compounds detected was based on the accurate *m/z* of the (de)protonated molecule and at least two fragment ions, together with the agreement in retention time (deviation lower than ± 2.5%). Both the fragment ions and retention times, used as references were derived from the metabolism experiments. Up to nine OMs were detected in the water samples, opposite to parent omeprazole that was not found in any of the samples (Table 2). OM10 was the most frequently detected compound, as it was present in 21 out of 25 WW, and in 11 out of 27 SW samples analyzed. OM7c, OM7d, OM11a, OM13, OM14a, and OM14b were also found in all types of water matrices, while OM5 was only detected in SW (21%) and EWW (10%) samples. Finally, OM7e was found in 3 SW samples (11%).

3.4. UHPLC-MS/MS analysis

In order to confirm the presence of OMs in the water samples, the same water extracts were re-analyzed by UHPLC-(ESI)-MS/MS with triple quadrupole, applying the same LC conditions used in the UHPLC-QTOF measurements in metabolism experiments. The higher sensitivity of triple quadrupole was expected to facilitate the detection of those OMs that were present at low concentrations in the water samples. The 24 OMs resulting from the metabolism study and parent omeprazole were included in the method. For each compound, two transitions were selected based on fragment ions observed in the QTOF experiments (Table S1). Apart from the presence of a chromatographic peak at the two transitions acquired, the identity of the findings was confirmed by calculating the

peak area ratio between the quantification "Q" and the confirmation "q" transitions, which was compared with that of the "reference compound" (urine vials with the highest concentration of the OM). A finding was considered positive when experimental ion ratios were within the tolerance range (Commission Decision 2002). The agreement in retention time (deviation lower than ± 2.5%) with the "reference compound" was also required.

Up to 14 OMs were detected and their identity confirmed by QqQ (Table 2). Eleven of these OMs (OM4, OM5, OM7c, OM7d, OM7e, OM8b, OM10, OM11a, OM13, OM14a, and OM14b) were found in all types of water matrices analyzed. Similar to QTOF analysis, unchanged omeprazole was not found in any sample. Nevertheless, it is interesting to note that one of its isomers, OM11a, was detected in 80% IWW, 90% EWW and 30% SW samples. This fact reveals the importance of good chromatographic separation to avoid confusion between these two compounds as they share one of the transitions (346.1 > 198.1). Therefore, in order to reduce false positives of omeprazole the acquisition of additional specific transitions for each compound (see Table S1) and satisfactory chromatographic separation is of high relevance. This compound might be the same detected in wastewater by Gómez-Ramos et al. (2011), after performing an XIC at the exact mass of omeprazole. OM10 was the most abundant in terms of MS arbitrary units, being consistent with the results of QTOF analysis. This compound was the most frequently detected metabolite in WW (100%) and SW (48%). Five of the OMs (OM3, OM4, OM8a, OM8b, and OM8c) were only found by LC-MS/MS analysis, due to its higher sensitivity compared to QTOF.

It is noteworthy that up to eight OMs were detected in 90–100% of effluent wastewater samples. These OMs were also present in influent wastewater, although some of them at lower frequency. Surely, the higher complexity and strong signal suppression due to matrix effects in the influent made the detection of OMs more problematic in comparison with effluent wastewater. It is also relevant the detection of up to seven OMs in around 30% of the surface water samples.

Table 2. Summary of the results obtained in the investigation of omeprazole metabolites in wastewater and surface water samples using QTOF and QqQ MS analyzers.

	% positive findings					
	LC-QTOF MS (retrospective)			LC-MS/MS QqQ		
Compound name	IWW (n=15)	EWW (n=10)	SW (n=27)	IWW (n=15)	EWW (n=10)	SW (n=27)
Omeprazole	-	-	-	-	-	-
OM1	-	-	-	-	-	-
OM2a	-	-	-	-	-	-
OM2b	-	-	-	-	-	-
OM2c	-	-	-	-	-	-
OM3	-	-	-	7	-	-
OM4	-	-	-	20	60	19
OM5	-	10	21	60	100	33
OM6	-	-	-	-	-	-
OM7a	-	-	-	-	-	-
OM7b	-	-	-	-	-	-
OM7c	7	80	19	60	100	30
OM7d	20	90	26	47	100	26
OM7e	-	-	11	13	90	15
OM8a	-	-	-	13	40	-
OM8b	-	-	-	33	70	15
OM8c	-	-	-	-	30	11
OM9	-	-	-	-	-	-
OM10	80	90	41	100	100	48
OM11a	20	10	4	80	90	30
OM11b	-	-	-	-	-	-
OM13	13	20	21	20	80	37
OM14a	13	90	22	100	100	37
OM14b	20	90	26	100	100	41

As an illustrative example, Fig. 3 shows selected UHPLC-MS/MS chromatograms for the most abundant OMs (OM5, OM10, OM7c,d,e, OM14a,b) detected in an effluent wastewater. As can be seen, experimental ion ratios did not exceed the maximum deviation allowed for any of the OMs detected, with all deviations being below 15%. OM10 and OM14b presented the highest responses, with average areas of 10,000 and

8000 a.u., respectively. This might reveal that they were the most relevant compounds in terms of concentration, but however quantification could not be performed due to the lack of reference standards.

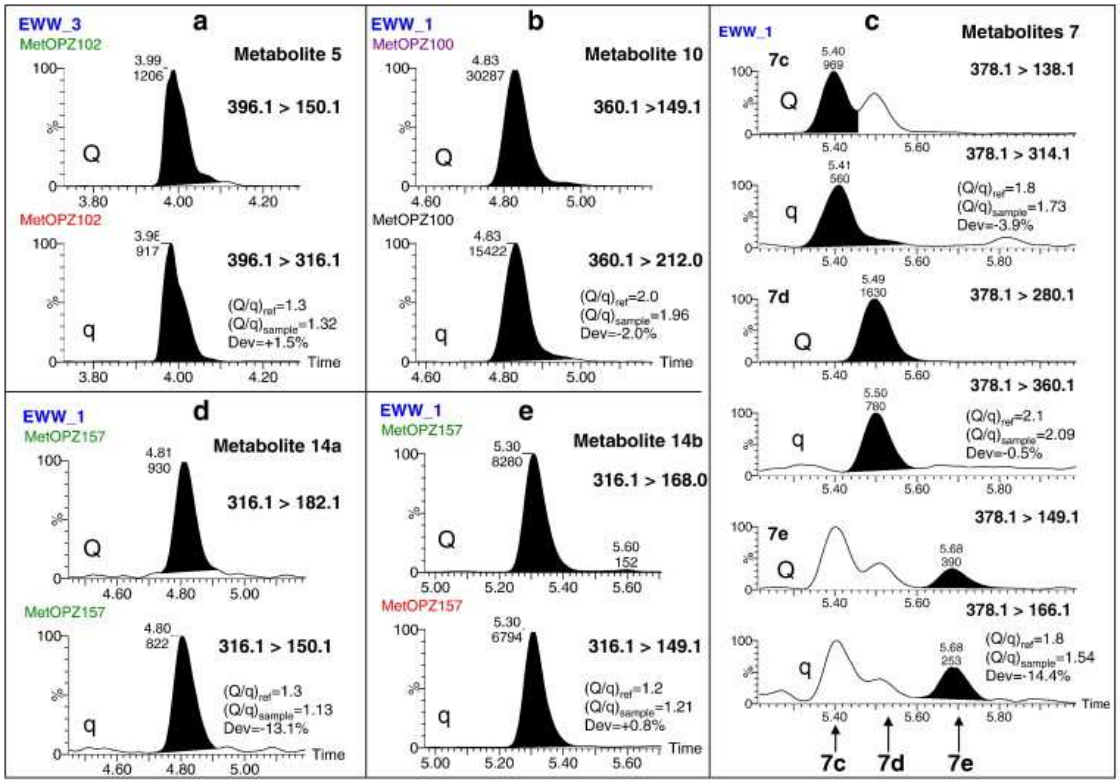

Fig. 3. UHPLC-MS/MS chromatograms for the omeprazole metabolites (a) OM5, (b) OM10, (c) OM7c–7e, (d) OM14a and (e) OM14b, in effluent wastewater.

4. CONCLUSIONS

In this work, urinary omeprazole metabolites have been investigated by UHPLC-QTOF MS. A total of twenty-four OMs were identified in urine samples of three volunteers who participated in this study, while parent omeprazole was present only at very low concentrations. OM11a was the most abundant compound. This OM is an omeprazole isomer and shares the fragment ion at *m*/*z* 198.0589 commonly used for the determination of omeprazole in LC-MS/MS methods. The loss of [$^{\bullet}$SH] radical deduced by TOF MS spectra in most of the OMs detected, has been crucial for justifying and suggesting possible chemical structures.

After UHPLC-QTOF MS analysis of 52 water samples, nine OMs were detected in surface water and wastewater samples, with OM10 being the most frequently found in wastewater (84% of the samples) and in surface water (41%). The results were confirmed by UHPLC-MS/MS analysis using a triple quadrupole analyzer, which superior sensitivity allowed to detect up to fourteen OMs. Unchanged omeprazole was not found in any sample; nevertheless, its isomer OM11a was detected in several samples.

In the light of the results obtained in the present work, it seems evident that monitoring unchanged omeprazole is not the best option to investigate the impact of this widely consumed pharmaceutical in the aquatic ecosystem. Instead, it is recommended to focus the research on the most abundant OMs identified in this work, i.e., those named as OM5, OM7c, OM7d, OM10, OM11a, OM14a, and OM14b, when monitoring omeprazole in urban wastewater and also in surface water. Obviously, it would be necessary to perform absolute configuration on the relevant compounds by NMR to subsequently enable synthesis of reference compounds to perform quantitative studies, but that is beyond the present study. In addition to the OMs proposed in this paper, omeprazole sulfide – a transformation product resulting from hydrolysis of omeprazole that has been previously reported (Boix et al., 2013) – should also be included to have a realistic overview of the omeprazole impact on the aquatic ecosystem.

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REFERENCES

T. Andersson, J.O. Miners, M.E. Veronese, W. Tassaneeyakul, W. Tassaneeyakul, U.A. Meyer *et al.* Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism Br. J. Clin. Pharmacol., 36 (1993), pp. 521–530.

A. Awasthi, M. Razzak, R. Al-Kassas, D.R. Greenwood, J. Harvey, S. Garg Isolation and characterization of degradation products of moxidectin using LC, LTQ FT-MS, H/D exchange and NMR Anal. Bioanal. Chem., 404 (2012), pp. 2203–2222.

J.P. Besse, C. Kausch-Barreto, J. Garric Exposure assessment of pharmaceuticals and their metabolites in the aquatic environment: application to the French situation and preliminary prioritization Hum. Ecol. Risk Assess, 14 (2008), pp. 665–695.

J.P. Besse, J.F. Latour, J. Garric Anticancer drugs in surface waters. What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? Environ. Int., 39 (2012), pp. 73–86.

C. Boix, M. Ibáñez, J.V. Sancho, W.M.A. Niessen, F. Hernández Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry: degradation experiments J. Mass Spectrom., 48 (2013), pp. 1091-1100.

A.T. Bruni, M.M.C. Ferreira Theoretical study of omeprazole behavior: racemization barrier and decomposition reaction Int. J. Quantum Chem., 108 (2008), pp. 1097–1106.

P. Calza, C. Medana, E. Padovano, V. Giancotti, C. Baiocchi Identification of the unknown transformation products derived from clarithromycin and carbamazepine using liquid chromatography/high-resolution mass spectrometry Rapid Commun. Mass Spectrom., 26 (2012), pp. 1687–1704.

S. Castiglioni, R. Fanelli, D. Calamari, R. Bagnati, E. Zuccato Methodological approaches for studying pharmaceuticals in the environment by comparing predicted and measured concentrations in River Po, Italy Regul. Toxicol. Pharmacol., 39 (2004), pp. 25–32.

H. Chen, H. Wang, Y. Chen, H. Zhang Liquid chromatography-tandem mass spectrometry analysis of anisodamine and its phase I and II metabolites in rat urine J. Chromatogr. B Anal. Technol. Biomed. Life Sci., 824 (2005), pp. 21–29.

E.W. Chung, E.N.M. Ho, D.K.K. Leung, F.P.W. Tang, K.C.H. Yiu, T.S.M. Wan Detection of anti-ulcer drugs and their metabolites in horse urine by liquid chromatography - mass spectrometry Chromatographia, 59 (2004), pp. S29–S38.

Commission Decision 2002/657/CE of 12 August 2002 Implementing Council Directive 6/23/EC Concerning the Performance of Analytical Methods of Interpretation of Results (2002).

O. Corcoran, J.K. Nicholson, E.M. Lenz, F. Abou-Shakra, J. Castro-Perez, A.B.. Sage *et al.* Directly coupled liquid chromatography with inductively coupled plasma mass spectrometry and orthogonal acceleration time-of-flight mass spectrometry for the identification of drug metabolites in urine: application to diclofenac using chlorine and sulfur detection Rapid Commun. Mass Spectrom., 14 (2000), pp. 2377–2384.

M. DellaGreca, M.R. Iesce, L. Previtera, M. Rubino, F. Temussi, M. Brigante Degradation of lansoprazole and omeprazole in the aquatic environment Chemosphere, 63 (2006), pp. 1087–1093.

R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández Building an empirical mass spectra library for screening of organic pollutants by ultra-high-pressure liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry Rapid Commun. Mass Spectrom., 25 (2011), pp. 355–369.

M. Espinosa Bosch, A.J. Ruiz Sánchez, F. Sánchez Rojas, C. Bosch Ojeda Analytical methodologies for the determination of omeprazole: an overview J. Pharm. Biomed. Anal., 44 (2007), pp. 831–844.

M. Gómez-Ramos, A. Pérez-Parada, J.F. García-Reyes, A.R. Fernández-Alba, A. Agüera Use of an accurate -mass database for the systematic identification of transformation products of organic contaminants in wastewater effluents J. Chromatogr. A, 1218 (2011), pp. 8002–8012.

S. González Alonso, M. Catalá, R.R. Maroto, J.L.R. Gil, Á.G. de Miguel, Y. Valcárcel Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain) Environ. Int., 36 (2010), pp. 195–201.

E. Gracia-Lor, J.V. Sancho, F. Hernández Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry J. Chromatogr. A, 1217 (2010), pp. 622–632.

F. Hernández, J.V. Sancho, O.J. Pozo An estimation of the exposure to organophosphorus pesticides through the simultaneous determination of their main metabolites in urine by liquid chromatography-tandem mass spectrometry J Chromatogr. B Anal. Technol. Biomed. Life Sci., 808 (2004), pp. 229–239.

F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry Anal. Chim. Acta, 684 (2011), pp. 87–97.

F. Hernández, M. Ibáñez, E. Gracia-Lor, J.V. Sancho Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater J. Sep. Sci., 34 (2011), pp. 3517–3526.

M.D. Hernando, M.J. Gómez, A. Agüera, A.R. Fernández-Alba LC-MS analysis of basic pharmaceuticals (beta-blockers and anti-ulcer agents) in wastewater and surface water Trends Anal. Chem., 26 (2007), pp. 581–594.

G. Hopfgartner, I.V. Chernushevich, T. Covey, J.B. Plomley, R. Bonner Exact mass measurement of product ions for the structural elucidation of drug metabolites with a tandem quadrupole orthogonal-acceleration time-of-flight mass spectrometer J. Am. Soc. Mass Spectrom., 10 (1999), pp. 1305–1314.

M. Ibáñez, J.V. Sancho, Ó.J. Pozo, F. Hernández Use of quadrupole time-of-flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure Anal. Chem., 76 (2004), pp. 1328–1335.

M. Ibáñez, J.V. Sancho, Ó.J. Pozo, F. Hernández Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case study Anal. Bioanal. Chem., 384 (2006), pp. 448–457.

M. José Gómez, O. Malato, I. Ferrer, A. Agüera, A.R. Fernández-Alba Solid-phase extraction followed by liquid chromatography-time-of-flight- mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study J. Environ. Monit., 9 (2007), pp. 719–729.

H. Kanazawa, A. Okada, Y. Matsushima, H. Yokota, S. Okubo, F. Mashige *et al.* Determination of omeprazole and its metabolites in human plasma by liquid chromatography–mass spectrometry J. Chromatogr. A, 949 (2002), pp. 1–9.

J. Macek, J. Klíma, P. Ptáček Rapid determination of omeprazole in human plasma by protein precipitation and liquid chromatography-tandem mass spectrometry J Chromatogr. B Anal. Technol. Biomed. Life Sci., 852 (2007), pp. 282–287.

S. Ortiz de García, G. Pinto Pinto, P. García Encina, R. Irusta Mata Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain Sci. Total Environ., 444 (2013), pp. 451–465.

A. Petsalo, M. Turpeinen, O. Pelkonen, A. Tolonen Analysis of nine drugs and their cytochrome P450-specific probe metabolites from urine by liquid chromatography–tandem mass spectrometry utilizing sub 2 μm particle size column J. Chromatogr. A, 1215 (2008), pp. 107–115.

R.S. Plumb, K.A. Johnson, P. Rainville, B.W. Smith, I.D. Wilson, J.M. Castro-Perez *et al.* UPLC/MSE; a new approach for generating molecular fragment information for biomarker structure elucidation Rapid Commun. Mass Spectrom., 20 (2006), pp. 1989–1994.

Prescription data: IT del Sistema Nacional de Salud Volumen 35, No 4/2011 http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf.

N.L. Rezk, K.C. Brown, A.D.M. Kashuba A simple and sensitive bioanalytical assay for simultaneous determination of omeprazole and its three major metabolites in human blood plasma using RP-HPLC after a simple liquid-liquid extraction procedure J. Chromatogr. B Anal. Technol. Biomed. Life Sci, 844 (2006), pp. 314–321.

K.L. Rost, J. Brockmöller, F. Esdorn, I. Roots Phenocopies of poor metabolizers of omeprazole caused by liver disease and drug treatment J. Hepatol., 23 (1995), pp. 268–277.

Q. Song, W. Naidong Analysis of omeprazole and 5-OH omeprazole in human plasma using hydrophilic interaction chromatography with tandem mass spectrometry (HILIC–MS/MS)—eliminating evaporation and reconstitution steps in 96-well liquid/liquid extraction J. Chromatogr. B, 830 (2006), pp. 135–142.

T. Ternes, M. Bonerz, T. Schmidt Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography–electrospray tandem mass spectrometry J. Chromatogr. A, 938 (2001), pp. 175–185.

M. Thevis, A. Thomas, I. Möller, H. Geyer, J.T. Dalton, W. Schänzer Mass spectrometric characterization of urinary metabolites of the selective androgen receptor modulator S-22 to identify potential targets for routine doping controls Rapid Commun. Mass Spectrom., 25 (2011), pp. 2187–2195.

A.L.N. Van Nuijs, I. Tarcomnicu, W. Simons, L. Bervoets, R. Blust, P.G. Jorens *et al.* Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater Anal. Bioanal. Chem., 398 (2010), pp. 2211–2222.

L. Weidolf, N. Castagnoli Jr. Study of the electrospray ionization mass spectrometry of the proton pump inhibiting drug omeprazole Rapid Commun. Mass Spectrom., 15 (2001), pp. 283–290.

E. Zuccato, S. Castiglioni, R. Fanelli Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment J. Hazard. Mater., 122 (2005), pp. 205–209.

SUPPLEMENTARY INFORMATION

Identification of new omeprazole metabolites in wastewaters and surface waters

1. Reagents and chemicals

Omeprazole was purchased from Sigma-Aldrich (Steinheim, Germany). 4-hydroxy omeprazole sufide and 5-hydroxy omeprazole were obtained from Toronto Research Chemicals (Ontario, Canada). Solutions of 50 mg L^{-1}, stored in amber glass bottles at -18 ^{o}C, were used for LC-MS analysis.

Analytical grade acetic acid, ammonium acetate, sodium hydroxide (99%), hydrochloric acid (37%) and formic acid (98-100%), as well as HPLC-grade methanol and acetonitrile were purchased from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water in a Milli-Q Gradient A10 (Millipore, Bedford, MA, USA).

The enzyma β-D-glucuronidase as well as leucine enkephaline, used as lock mass, were purchased from Sigma-Aldrich.

For analysis of excretion in urine, Omeprazole (Ratiopharm EFG - Spain) in capsules of 20 mg dose was purchased from local chemists.

2. Instrumentation

2.1 UHPLC-QTOF MS

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 chromatographic separation was performed using an Acquity UPLC BEH C18 1.7 μm, 100 mm × 2.1 mm analytical column (Waters) at a flow rate of 300 μL min^{-1}. The mobile phases used were H_2O and MeOH, both 0.01% HCOOH. The percentage of organic modifier was changed linearly as follows: 0 min, 10%; 9 min, 90%; 11 min, 90%; 11.10 min, 10%; 14 min, 10%. Nitrogen (from a nitrogen generator) was

used as the drying gas and nebulizing gas. The gas flow was set at 600 L h^{-1}. TOF MS resolution was approximately 10.000 at full width half maximum (FWHM) at *m/z* 556. MS data were acquired over an *m/z* range of 50–1000. The microchannel plate (MCP) detector potential was set to 1950 V. The capillary voltage was set to 3.5 kV and -3.0 kV in positive and negative ion modes, respectively. A cone voltage of 25 V was used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 350 °C and the source temperature to 120 °C. The column temperature was set to 40 °C.

Calibrations were conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.05 M NaOH 5% HCOOH diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 10 µL min^{-1}. For automated accurate mass measurement, the lock-spray probe was used, using as lockmass a solution of leucine enkephalin (2 µg mL^{-1}) in acetonitrile:water (50:50) at 0.1% HCOOH pumped at 30 µL min^{-1} through the lock-spray needle. Cone voltages of 80 and 70 V were selected in positive and negative ion modes, respectively, to obtain adequate signal intensity for this compound (~ 400 counts). The protonated (*m/z* 556.2771) or deprotonated (*m/z* 554.2615) molecule of leucine enkephalin was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time.

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 eV to 40 eV in order to promote fragmentation. The LE and HE functions settings were for both a scan time of 0.15 s and an inter-scan delay of 0.05 s. This approach enables the simultaneous acquisition of both parent (deprotonated or protonated molecules) and fragment ions in a single injection. For MS/MS experiments, a collision energy ramp ranging from 15 eV to 40 eV was also used.

2.2 UHPLC-MS/MS QqQ

A Waters Acquity UPLC system was interfaced to a triple quadrupole mass spectrometer (TQD, Waters) with an orthogonal Z-spray-electrospray interface. 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^{-1} and 1200 L h^{-1}, respectively. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a pressure of $2 \cdot 10^{-3}$ mbar in the T-Wave collision cell. A capillary voltage of 3.5 kV in positive ionization mode was applied. The interface temperature was set to 500 ºC and the source temperature to 120 ºC. A dwell time of 0.015 s was selected. Cone and collision energies could not be optimized as reference standards were not available. Instead, typical values of 30 V and 25 eV, respectively, were selected. Seventeen TPs as well as the omeprazole itself were monitored, acquiring two transitions per compound based on the fragment ions observed in the TPs identified along degradation experiments. Regarding UHPLC conditions, the same gradient and analytical column indicated in LC-QTOF MS analysis were applied.

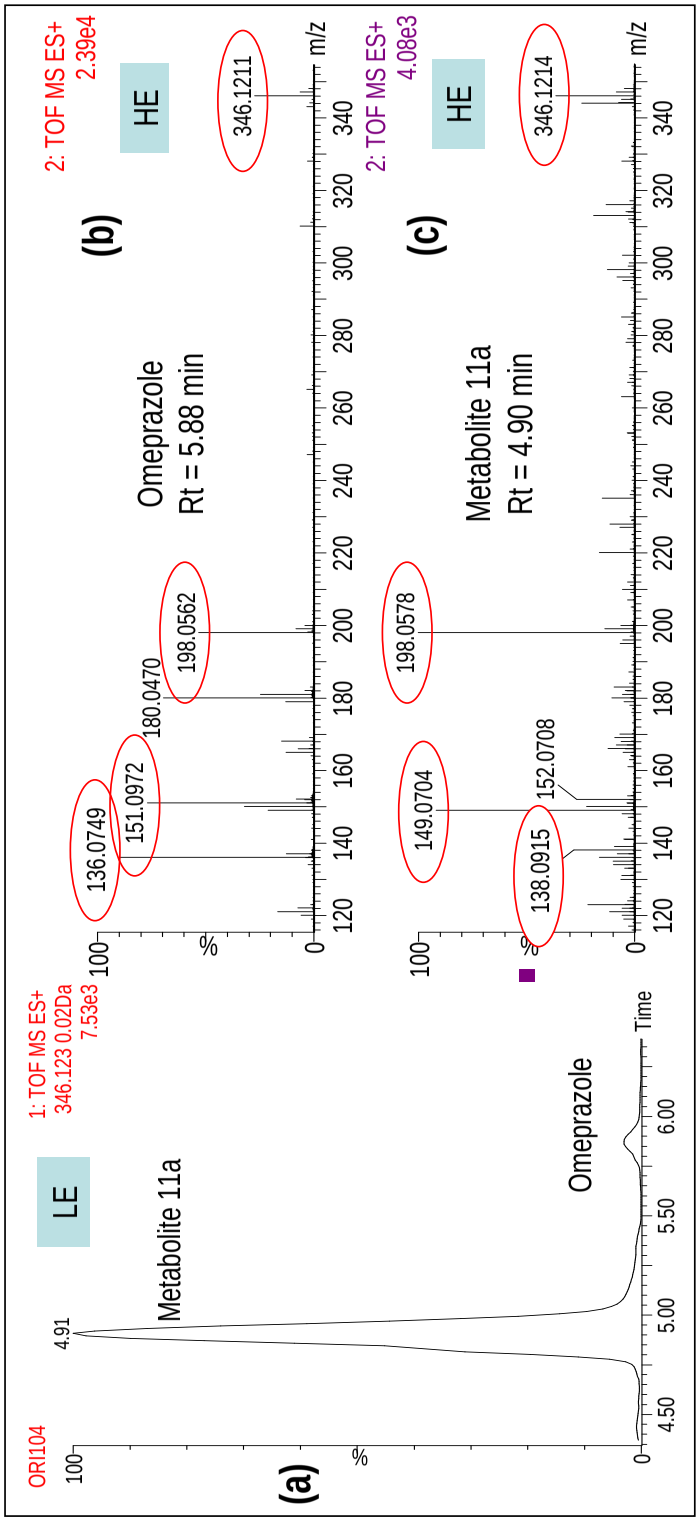

Figure S1. (a) LE nw-XIC for *m/z* 346.1225. HE MS spectra for (b) omeprazole and (c) OM11a detected in urine sample after 3.5 hours of drug administration.

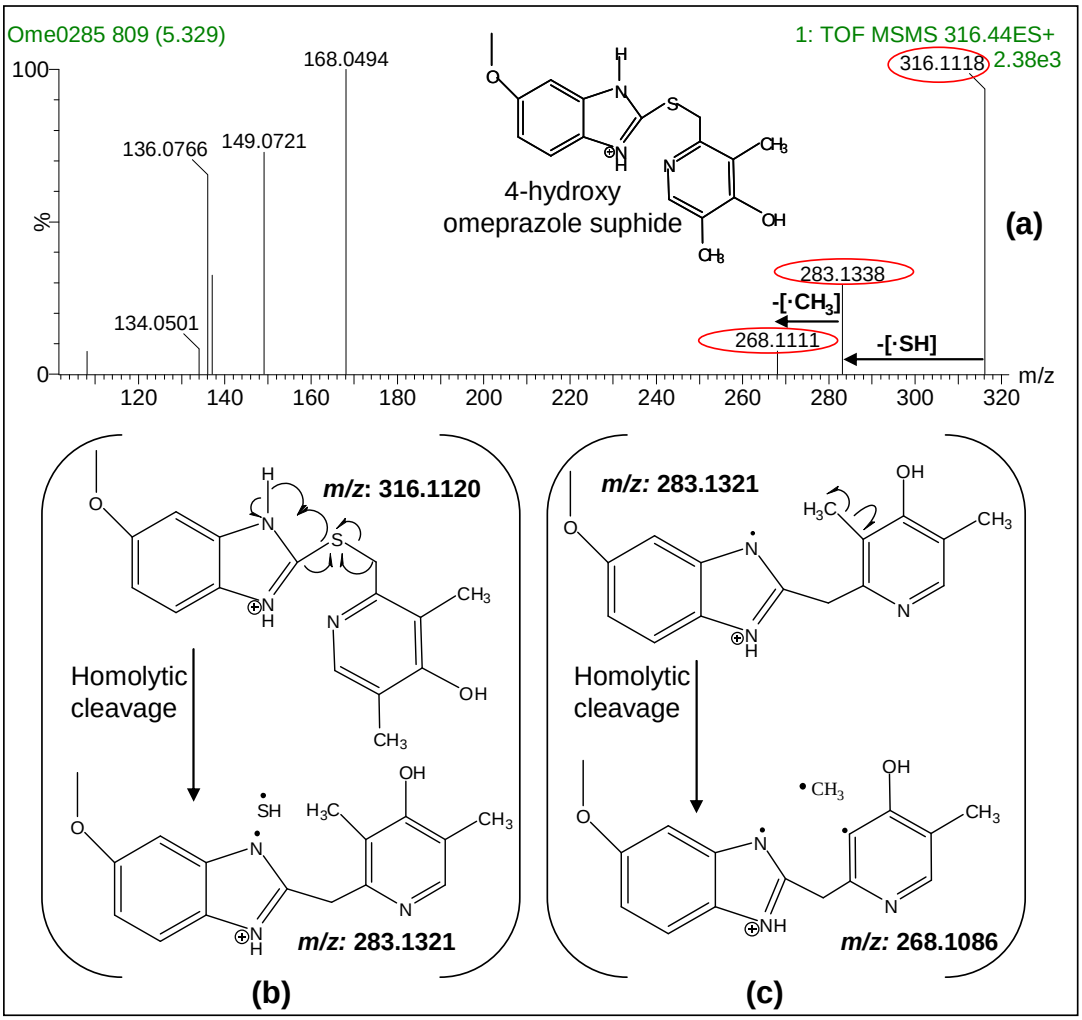

Figure S2. (a) MS/MS spectra of 4-hydroxy omeprazole sulfide. Possible fragmentation pathway for observed radical losses of (b) [$^{\bullet}$HS] and (c) [$^{\bullet}$CH$_3$].

1. Elucidation of Metabolite 11a

Figure S3 illustrates the elucidation of metabolite 11a. The accurate mass of the protonated molecule of this compound, with retention time 4.93 min, was measured to be *m/z* 346.1202 (Figure S3a). This mass differed -2.3 mDa from *m/z* 346.1225, corresponding to an elemental composition of $[C_{17}H_{20}N_3O_3S]^+$. This metabolite has the same exact mass than omeprazole, but elutes at different retention time and evidences different HE MS spectrum. However, they share an important fragment at *m/z* 198.0589.

The presence of the fragment at *m/z* 313.1374 supported the aforementioned loss of [$^{\bullet}$SH] radical, and the fragment at *m/z* 298.1180 would be related with the subsequent loss of [$^{\bullet}CH_3$] radical. The fragment at *m/z* 198.0581 would correspond to the loss of the benzimidazole ring (-$C_8H_8N_2O$). Fragment ions at *m/z* 166.0871 $[C_9H_{12}NO_2]^+$ and *m/z* 152.0699 $[C_8H_{10}NO_2]^+$ would justify the presence of an extra oxygen atom in the pyridine ring (Figure S3b). In the light of our data, the hydroxylation is proposed to occur in the methoxy group (position 4), in the methyl radical (position 5) or in position 6 of this ring. It seems not possible the hydroxylation in position 3 (Figure S3a) due to the observed loss of the methyl radical in this position, as explained in Figure S2c.

2. Elucidation of Metabolite 10

Figure S4 illustrates the elucidation process for metabolite 10. The accurate mass of the protonated molecule, with retention time 4.78 min, was found to be *m/z* 360.1019, which corresponds to an elemental composition $[C_{17}H_{18}N_3O_4S]^+$ with a mass error of +0.1 mDa. This compound presents one oxygen atom more than omeprazole. As in the previous case, the losses of [$^{\bullet}$SH] and [$^{\bullet}CH_3$] radicals, giving fragments at *m/z* 327.1177 and 312.0987, respectively, supported the chemical structure suggested. Thus, the reduction of the sulfoxide group of omeprazole would take place, keeping intact the methyl radical at position 3, followed by a hydroxylation and oxidation of the reduced specie.

The two most abundant fragments at *m/z* 212.0375 (-0.6 mDa) and 149.0708 (-0.7 mDa) would correspond to $[C_9H_{10}NO_3S]^+$ and $[C_8H_9N_2O]^+$ respectively, suggesting that the oxidation reactions occurred in the pyridine ring, rather than in the benzimidazole ring. According to the fragment *m/z* 180.0654 $[C_9H_{10}NO_3]^+$ (+0.7 mDa), the oxidation and hydroxylation might occur in four sites of the pyridine ring: on the position 6, on the methyl radicals (positions 2, 5) or on the methoxy group (position 4) (Figure S4c). Taking into account the presence of the fragment *m/z* 312.0987, the oxidation and hydroxylation on the 3-methyl radical seems not feasible (see Figure S2c), as shown in Figure S4b.

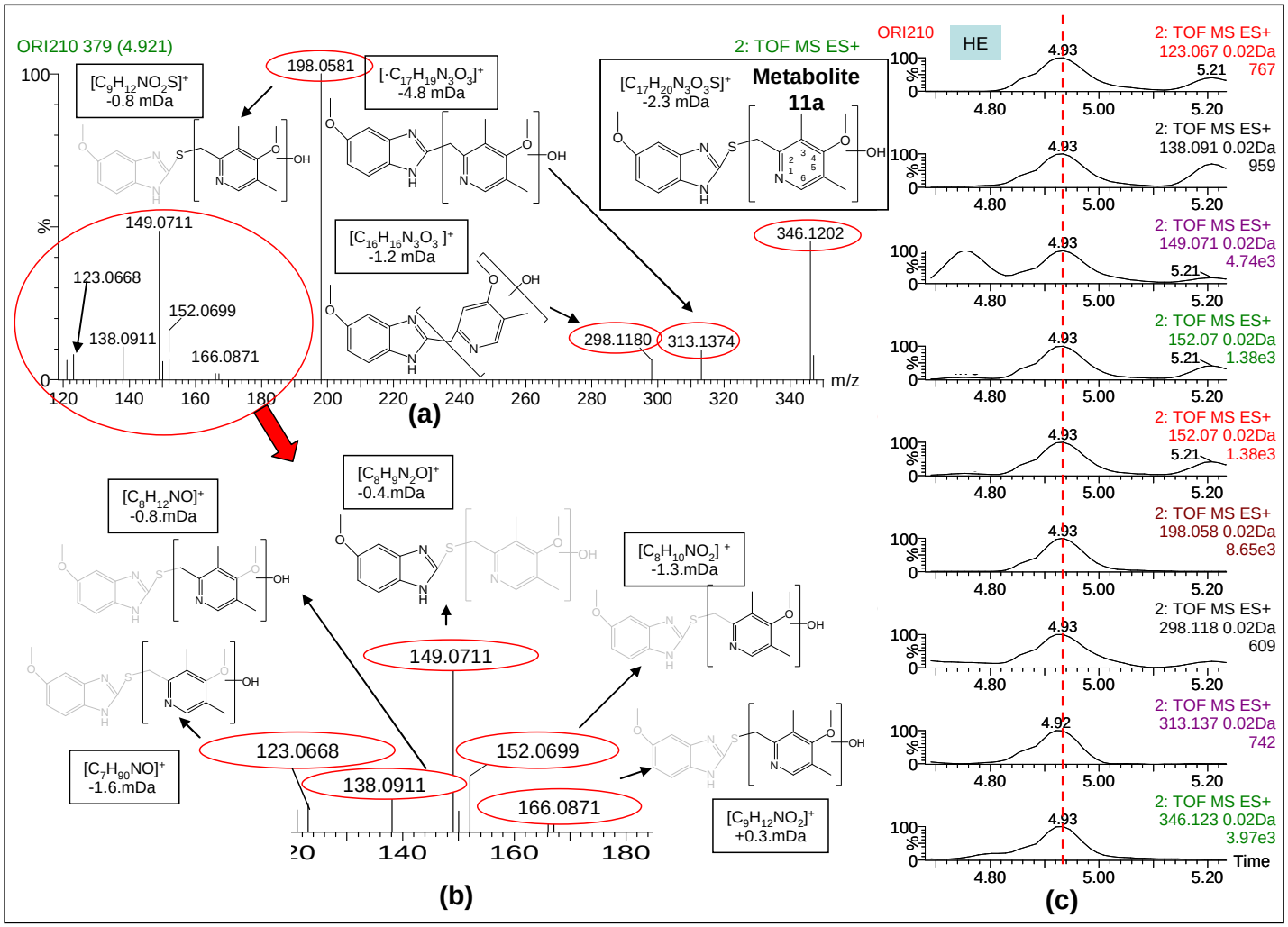

Figure S3. Elucidation of Metabolite 11a. (a) HE spectra and possible structure of metabolite 11a; (b) possible fragment ion structures proposed; and (c) nw-XICs of specific fragments obtained in HE.

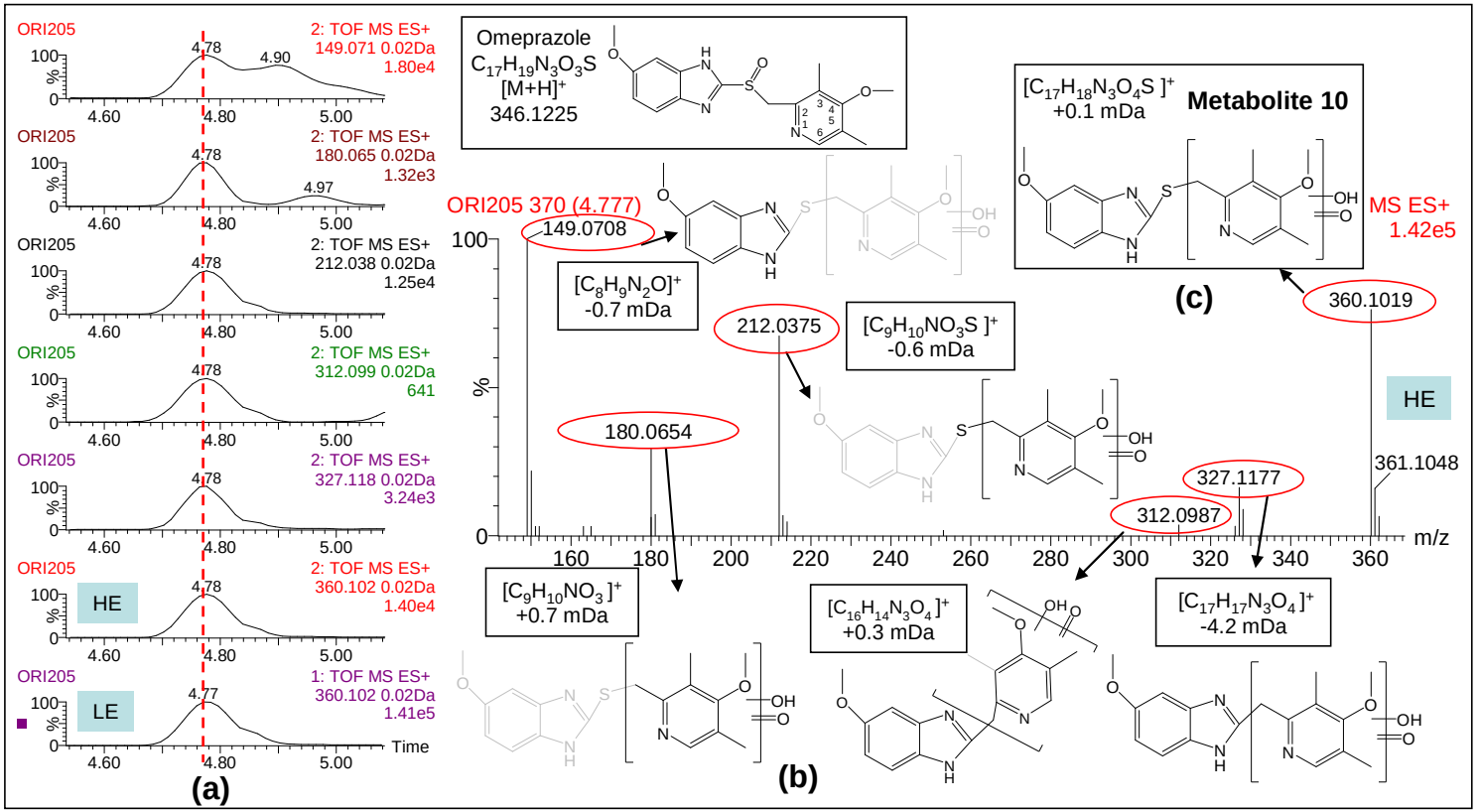

Figure S4. Elucidation of Metabolite 10. (a) nw-XICs for the protonated molecule (LE function) and fragment ions (HE function). (b) HE MS spectra and possible structures of metabolite 10 and their fragments.

Table S1. MS parameters selected in the analysis of omeprazole metabolites by QTOF and QqQ mass analyzers.

			QTOF conditions		QqQ conditions			
Compound name	Retention time (min)	Ionization mode	Elemental Composition	Theoretical Mass *(m/z)*	Parent *(m/z)*	Product ion 1 (Q)	Product ion 2 (q)	Q/q ratio
Omeprazole	5.98	ESI+	$C_{17}H_{20}N_3O_3S^+$	346.1225	346.1	136.1	198.1	1.5
OM1	3.13	ESI+	$C_{20}H_{25}N_4O_5S_2^+$	465.1266	465.1	298.1	344.1	4.4
OM2a	3.59	ESI+	$C_{23}H_{28}N_3O_9S^+$	522.1546	522.2	346.1	298.1	>1000
OM2b	4.48	ESI+	$C_{23}H_{28}N_3O_9S^+$	522.1546	522.2	328.1	489.2	2.6
OM2c	4.82	ESI+	$C_{23}H_{28}N_3O_9S^+$	522.1546	522.2	328.1	198.1	12.8
OM3	3.60	ESI+	$C_{16}H_{18}N_3O_4S^+$	348.1018	348.1	214.1	152.1	10.2
OM4	3.73	ESI+	$C_{16}H_{18}N_3O_3S^+$	332.1069	332.1	198.1	135.0	3.5
OM5	3.94	ESI+	$C_{16}H_{18}N_3O_5S_2^+$	396.0688	396.1	150.1	316.1	1.3
OM6	4.20	ESI+	$C_{17}H_{18}N_3O_4^+$	328.1297	328.1	120.1	195.1	2.3
OM7a	4.43	ESI+	$C_{17}H_{20}N_3O_5S^+$	378.1124	378.1	168.1	212.0	1.3
OM7b	4.90	ESI+	$C_{17}H_{20}N_3O_5S^+$	378.1124	378.1	149.1	328.1	27.1
OM7c	5.23	ESI+	$C_{17}H_{20}N_3O_5S^+$	378.1124	378.1	138.1	314.2	1.8
OM7d	5.32	ESI+	$C_{17}H_{20}N_3O_5S^+$	378.1124	378.1	280.1	360.1	2.1
OM7e	5.55	ESI+	$C_{17}H_{20}N_3O_5S^+$	378.1124	387.1	149.1	166.1	1.8
OM8a	4.50	ESI+	$C_{17}H_{20}N_3O_4S^+$	362.1175	362.1	149.1	344.1	1.1
OM8b	5.06	ESI+	$C_{17}H_{20}N_3O_4S^+$	362.1175	362.1	149.1	150.1	3.2
OM8c	5.06	ESI+	$C_{17}H_{20}N_3O_4S^+$	362.1175	362.1	150.1	120.1	3.3
OM9	4.48	ESI+	$C_{17}H_{20}N_3O_6S^+$	394.1073	394.1	376.1	149.1	1.9
OM10	4.78	ESI+	$C_{17}H_{18}N_3O_4S^+$	360.1018	360.1	149.1	212.0	2.0
OM11a	4.90	ESI+	$C_{17}H_{20}N_3O_3S^+$	346.1225	346.1	198.1	138.1	9.1
OM11b	4.52	ESI+	$C_{17}H_{20}N_3O_3S^+$	346.1225	346.1	198.1	313.1	7.1
OM12	4.59	ESI-	$C_{17}H_{18}N_3O_7S_2^-$	440.0586	440.1	410.0	312.0	1.5
OM13	3.35	ESI+	$C_{16}H_{16}N_3O_4S^+$	346.0862	346.1	135.1	212.0	2.4
OM14a	4.52	ESI+	$C_{16}H_{18}N_3O_2S^+$	316.1120	316.1	182.1	150.1	1.3
OM14b	5.01	ESI+	$C_{16}H_{18}N_3O_2S^+$	316.1120	316.1	168.0	149.1	1.2

3.4 Artículo Científico 4

INVESTIGATION OF OMEPRAZOLE AND VENLAFAXINE METABOLITES IN WASTEWATER MAKING USE OF HIGH RESOLUTION MASS SPECTROMETRY

(Paper in process)

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ABSTRACT

The present paper presents a comprehensive study on the occurrence of omeprazole and venlafaxine pharmaceuticals in Italian municipal influent wastewaters. The proton pump inhibitor omeprazole is scarcely detected in waters although this is highly consumed. Regarding venlafaxine, this antidepressant is commonly found in water samples, but also its main metabolite (O-desmethyl venlafaxine), usually detected at higher concentration levels. This is the interest on monitoring these compounds through the investigation of their main metabolites in influent wastewaters (IWWs). After a solid-phase extraction (SPE), 30 IWW samples from 10 Italian cities have been analyzed by high-performance liquid chromatography (HPLC) coupled to high resolution mass spectrometry (HRMS) with an Orbitrap analyzer. 23 omeprazole and 4 venlafaxine metabolites, identified in a previous work, were searched in a *"post-target"* way. Parent omeprazole was never present in any of the samples analyzed. Nevertheless, 6 of its metabolites were found in IWWs, being OM10 the omeprazole metabolite most frequently detected (100%). The isomer of omeprazole, OM11a, was found in 53% of the samples analyzed. Regarding venlafaxine, this drug and 2 of its metabolites were present in all of the samples analyzed. It is noteworthy, that O-desmethyl venlafaxine showed apparently higher concentrations levels (max. 7.1 ng/mL) than the parent compound (max. 3.1 ng/mL). Finally, the presence of these metabolites in Italian IWW was compared with those found in samples from Spain.

Keywords

Pharmaceuticals, influent/untreated wastewater, metabolites, liquid chromatography, high resolution mass spectrometry.

1. INTRODUCTION

According to the Centre for Promotion of Imports from Developing Countries (CBI, 2009), Italy was in 2007 the third largest pharmaceutical market in the European Union (EU), accounting for 12% of the total EU consumption. Omeprazole, for treatment of gastric diseases, and venlafaxine, used as antidepressant, are two of the most consumed pharmaceuticals in Italy and also around the world.

Pharmaceuticals can reach the environment through human excretion, improper disposal of unused and expired products and runoff from farm and livestock (Stuart et al., 2012; Zuccato et al., 2000). Among them, the excretion is the main source of contamination of the ecosystem, for this reason, a comprehensive literature search should be carried out to improve the knowledge of the metabolism and excretion of pharmaceuticals. Nevertheless, as Baker reported (Baker et al., 2014), this is a difficult process, given the lack of publication and the age of the publications making retrieval of information problematic.

Approximately 29% of a venlafaxine applied dose is excreted in the urine within 48 hours as the unconjugated metabolite O-desmethyl venlafaxine, whereas 5% of the amount is excreted as unchanged parent compound (Howell et al., 1993). For this reason, venlafaxine is found in wastewaters (Baker and Kasprzyk-Hordern, 2013; Gasser et al., 2012; Gracia-Lor et al., 2011; Van Nuijs et al., 2010; Writer et al., 2013; Boix et al., 2015) and also in environmental waters (Ferrer and Thurman, 2012; González Alonso et al., 2010; Gracia-Lor et al., 2011; Huntscha et al., 2012; Rúa-Gómez and Püttmann, 2012; Valcárcel et al., 2011; Boix et al., 2015), but its active metabolite, O-desmethyl venlafaxine, is usually found at higher concentration levels (Gasser et al., 2012; Rúa-Gómez and Püttmann, 2012; Writer et al., 2013).

The reported excretion rates for omeprazole (as intact parent) ranged from 0.01% (Besse et al., 2012) to 5% (Hernando et al., 2007), or even up to 30% (Ortiz de García et al., 2013). For this reason, and considering its reported high consumption, one might expect the presence of this pharmaceutical in waters. Nevertheless, this compound is almost not

detected in aquatic samples (Calamari et al., 2003; Castiglioni et al., 2006, 2005; Valcárcel et al., 2011; Van Nuijs et al., 2010; Zuccato et al., 2005; Boix et al., 2015). This fact together with the lack of information on omeprazol metabolism lead us to perform a human urinary metabolism study (Boix et al., 2014a). In this study, the parent omeprazole was scarcely excreted, but some new metabolites could be discovered in urine and afterwards retrospectively detected in Spanish wastewater (WW) and surface (SW) samples.

The majority of the multi-residual methods are focused on the parent pharmaceuticals, being limited the number of studies related to the investigation of omeprazole (Boleda et al., 2013; Gracia-Lor et al., 2014; Hernández et al., 2011) and venlafaxine (Ferrer and Thurman, 2012; Gasser et al., 2012; Huntscha et al., 2012) metabolites in waters.

The analytical determination of pharmaceuticals in waters is mostly based on liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) (Baker and Kasprzyk-Hordern, 2011; Dorival-García et al., 2013; González Alonso et al., 2010; Gracia-Lor et al., 2011; Senta et al., 2013; Van Nuijs et al., 2010; Zuccato et al., 2005) or ion trap (IT) (Gros et al., 2012; Sousa et al., 2011; Valcárcel et al., 2011) analyzers. High resolution mass spectrometry (HRMS) instruments, such as Orbitrap (Chitescu et al., 2012; Wode et al., 2012) and time-of-flight (TOF) (Díaz et al., 2013), are advanced analytical tools for the screening of organic contaminants and tentative identification and elucidation of unknowns. Thanks to the sensitive accurate-mass full-spectrum acquisition, these analyzers allow detecting compounds in a "*post-target*" way, without a previous optimization (Hernández et al., 2011), being a very useful approach when reference standards are not available (Bijlsma et al., 2013; Boix et al., 2013).

The objective of this work is to investigate the presence of omeprazole and venlafaxine metabolites (OMs and VMs) in Italian IWW collected from 10 different cities. A retrospective analysis has been performed by HPLC-LTQ-Orbitrap MS, searching for 23 omeprazole and 4 venlafaxine metabolites discovered in our previous works (Boix et al., 2014a; Boix et al., 2014b). In a second step, in order to confirm their identities the samples

were re-analyzed by MS and MS/MS in a sequentially acquisition mode. Finally, the results obtained in Italian waters were compared with those reported in Spanish samples.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Reference standard of omeprazole was obtained from Sigma-Aldrich (Steinheim, Germany). Venlafaxine hydrochloride was purchased from LGC Promochem (London, UK) and 4-hydroxy omeprazole sulphide, rac N-desmethyl venlafaxine and D,L-O-desmethyl venlafaxine were purchased from Toronto Research Chemicals (North York, Canada). Individual stock solutions of 500 mg/L were prepared in methanol.

HPLC-grade methanol (MeOH), acetonitrile (ACN) and formic acid (98%) were acquired from Fluka (Buchs, Switzerland). A Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA) was used to obtain the HPLC grade water.

Solid-phase extraction cartridges, Oasis MCX 60 mg, were purchased from Waters (Milford, MA, USA).

2.2. Instrumentation

Liquid Chromatography (HPLC)

Chromatography separation of the compounds was carried out on Agilent 1200 series capillary and nano pumps, using an Agilent Zorbax SB-C18 column (150 × 0.5 mm I.D., particle size 5 µm). A general gradient was used at a constant flow rate of 10 µL min-1, using ultrapure water with 0.1% FA (solvent A) and ACN (solvent B). The percentage of organic modifier (B) was changed linearly as follow: 0 min (2% B), 24 min (99% B), 34 min (99% B), 36 min (1% B), 38 min (1% B). The analytical column was re-equilibrated for 8 min. Analysis run time was 38 min. The sample injection volume was 2 µL of the sample extract.

High Resolution Mass Spectrometry (Orbitrap MS)

Analyses have been performed on a Thermo LTQ Orbitrap equipped with a Prosolia's Ommi Spray DESI ion source, used as a nano ES source. The conditions working in positive ionization mode were: source voltage 2.41 kV, heated capillary temperature 220 °C and capillary voltage 49 V. The equipment was operated in full-scan mode (60,000 resolution)

and in MS/MS mode (15,000 resolution) to confirm the identities. Products ions were generated in the LTQ trap at collision energy setting of 60% and using an isolation width of 2 Da. And the acquisition ranges were from 220 to 500 *m/z*.

2.3. Sample collection

Thirty influent wastewater samples were collected from 10 Italian wastewater treatment plants (WWTP): Bologna, Milano, Torino, Merano, Napoli, Palermo, Bari, Firenze, Verona and Roma, from October 2013 to January 2014. Samples were collected in amber glass bottles and stored at 4 °C for a maximum of 3 days, then were extracted and analyzed.

2.4. Extraction procedure (SPE)

Initially, the water samples were filtered (GF/A 1.6 µm and 0.45 µm). Then a solid-phase extraction was performed using a mixed reversed-phase/cation-exchange cartridges (Oasis-MCX). Briefly, wastewater samples (25 mL) were adjusted to a pH 2-2.5 with 37% HCl. The Oasis MCX cartridges were conditioned before use by washing with 5 mL of methanol, 3 mL of Milli-Q water, Samples were then passed through the cartridges under vacuum at a flow rate of 10 mL/min. Cartridges were vacuum dried for 5 min and eluted with 2 mL of methanol and 2 mL of a 2% ammonia solution in methanol (collected together). The eluates were pooled and dried under a nitrogen stream. Then, they were reconstituted with 100 µL of MilliQ water. Finally the samples were mixed (vortex) and centrifuged for 2 min. at 2500 rpm.

3. RESULTS AND DISCUSSION

The work performed in this paper is based on two of our previous studies. In the first one (Boix et al., 2014a), we carried out an omeprazole metabolism study, where up to 24 metabolites could be detected by UHPLC-QTOF MS in human urine samples. Then these compounds were searched in IWW, EWW and SW water samples. The second work (Boix et al., 2014b) was based on a biodegradation study of 5 highly consumed pharmaceuticals, being venlafaxine one of them. The presence of this pharmaceutical as well as 8 of its metabolites/transformation products (TPs) was investigated in different waters matrices.

Table 1SI shows the elemental composition, exact mass and fragment ions for omeprazole, venlafaxine and their proposed metabolites. These data were obtained by LC-ESI-QTOF MS and reported in our previous works (Boix et al., 2014a; Boix et al., 2014b). In the present study the same nomenclature than in the initial works has been used.

3.1. Analysis of the samples

3.1.1. Detection of metabolites

In order to test whether the parent omeprazole and venlafaxine and their metabolites were present in Italian water samples, a retrospective analysis was performed. For this purpose, 30 influent wastewater samples, previously analyzed by LC-Orbitrap, were investigated. Taking profit of the availability of untargeted accurate-mass full-spectrum data provided by this analyzer, 29 compounds (Table 1SI) were searched in the positive-ionization data collected from influent wastewater samples.

It is worth to notice that parent omeprazole was not found in any water sample. On the contrary, 14 out of 27 metabolites (10 of omeprazole and 4 of venlafaxine) could be presumably detected in several wastewaters, based on the presence of their accurate-mass protonated molecules, $[M+H]^+$ (Table 2SI).

The elution order of the compounds could be an additional and helpful tool to predict the assignation of the compounds. In that sense, taking into account the different mobile

phases, column and gradient, the elution order of the detected compounds was consistent with our data reported by QTOF MS. The retention time of omeprazole was the only exception, eluting before than expected (14.7 min). It could be due to the omeprazole was detected in solvent instead of in sample matrix.

Table 2SI summarizes the compounds tentatively detected in IWWs after a full-scan acquisition and their retention times using the chromatography conditions explained in section *2.2. Instrumentation*, as well as those previously reported (Boix et al., 2014a; Boix et al., 2014b).

However, fragment ions information must be necessary for a reliable identification of the metabolites. For this reason, a second LC-Orbitrap analysis based on MS/MS acquisitions was performed.

3.1.2. Confirmation of metabolites

A specific MS and MS/MS sequentially method was created for 16 tentative positive compounds. This methodology was applied to the same 30 influent wastewater samples. Confirmation of the identity of the compounds detected was based on the accurate *m/z* of the protonated molecule and at least two fragment ions (mass error <5 ppm), together with the agreement in retention time (deviation lower than ±2.5%) one samples to the others (Boix et al., 2013).

As shown in Table 1, omeprazole was not found in any of the samples analyzed; nevertheless 6 of its metabolites could be confirmed. As an illustrative example, Figure 1 shows selected LC-HRMS chromatograms for the OMs (OM7c-7d, OM10, OM11a, OM14b and OM13) detected in a Torino IWW. OM10 was the compound most frequently detected, being present in all the samples analyzed (100%). It was followed by OM7c (97%) and OM13 (77%). In addition, OM7d and OM14b were also detected and identified in 60% and 70% of the samples analyzed, respectively. The isomer of omeprazole (OM11a) was found in 16 out of 30 samples. Its presence, instead of the omeprazole, exposes the importance of good chromatographic separation to avoid confusion between these two

compounds, as they share two fragment ions (*m/z* 198.0584 and 149.0710). Therefore, in order to reduce false positives of omeprazole the acquisition of additional and/or specific fragment ions for each compound should be required. In order to illustrate the confirmation of positive findings, Figure 2 shows narrow window eXtracted Ion Chromatograms (nw-XICs) for protonated molecule of OM10, OM11a and OM13 and three of their fragment ions (two were enough to confirm a suspect compound) eluting at the same retention time and with mass errors below 5 ppm.

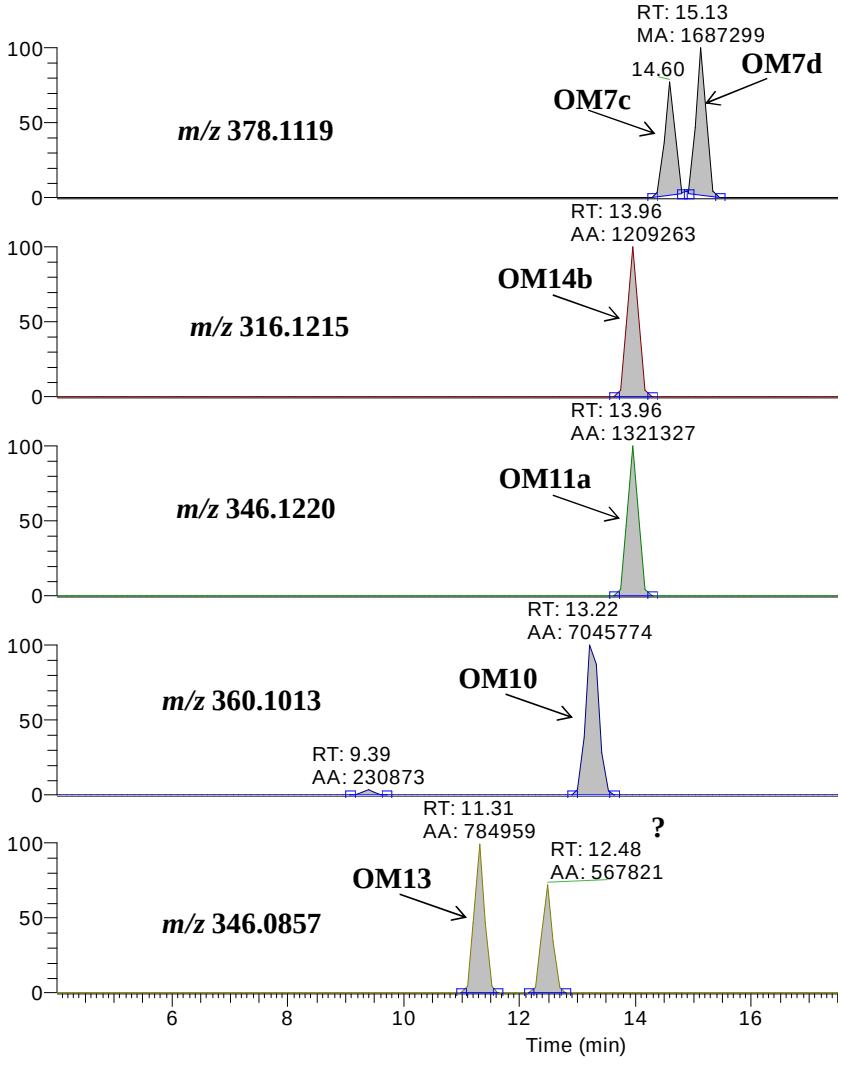

Figure 1. Positive findings of omeprazole metabolites in a Torino IWW sample.

Table 1. Pharmaceuticals and metabolites detected in Italian IWW by LC-Orbitrap.

Compound	Bologna	Milano	Torino	Merano	Napoli	Palermo	Bari	Firenze	Verona	Roma	% positive findings (n=30)
Omeprazole	-	-	-	-	-	-	-	-	-	-	-
OM7c	3/3	3/3	3/3	3/3	2/3	3/3	3/3	3/3	3/3	3/3	97
OM7d	3/3	1/3	2/3	0/3	1/3	3/3	3/3	2/3	3/3	3/3	70
OM10	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
OM11a	0/3	3/3	3/3	0/3	2/3	0/3	3/3	2/3	0/3	3/3	53
OM13	3/3	2/3	2/3	2/3	2/3	1/3	3/3	2/3	3/3	3/3	77
OM14b (4-OH omeprazole sulfide)	2/3	3/3	3/3	0/3	3/3	0/3	2/3	0/3	2/3	3/3	60
Venlafaxine	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
VB1a (O-Desmethyl-venlafaxine)	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100
V2	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	100

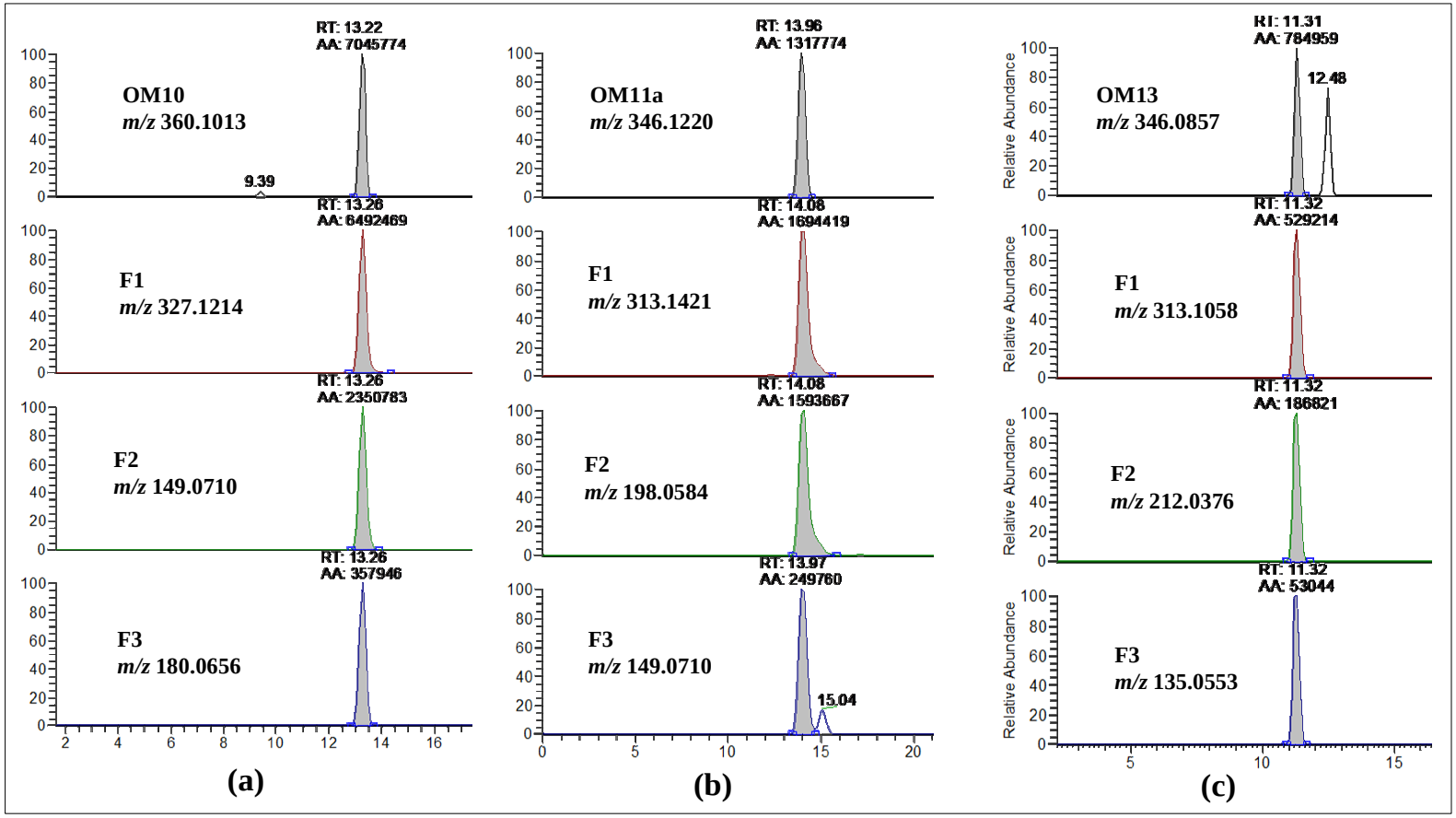

Figure 2. nw-XICs for protonated molecule of (a) OM10, (b) OM11a and (c) OM13 in the MS function, and three of their fragment ions in MS/MS functions.

OM10 and OM7d presented the highest responses, with average areas of 14,194,241 and 9,229,977 a.u., respectively. This might reveal that they were the most relevant compounds in terms of concentration, but however quantification could not be performed due to the lack of reference standards. Table 4SI shows a summary of the absolute areas of positive findings of omeprazole and venlafaxine detected in Italian IWW. The identity of OM14b could be additionally confirmed with a reference standard, corresponding to 4-OH omeprazole sulfide (standard available). Figure 1SI shows MS/MS spectra of the OM14b in a Bologna IWW and in the reference standard, showing up to three fragment ions. Moreover, a semi-quantitative estimation of 4-OH omeprazole sulfide in water samples was also done. Concentration values ranging from 0.1 to 1.0 ng/mL were obtained for the 30 samples analyzed. Torino showed the highest concentration level (medium concentration 0.8 ng/mL).

In the case of venlafaxine, the parent compound as well as 2 metabolites (VB1a and V2) were found in all IWW samples analyzed. These results are summarized in Table 1. As an example, Figure 3 shows nw-XICs of the 3 venlafaxine metabolites present in Firenze wastewater. It also illustrates the confirmation of a positive finding V2, sharing the *m/z* of the protonated molecule (258.1853) and four product ions (201.1274, 199.1118, 133.0648 and 157.0648) previously reported in (Boix et al., 2014b).

The presence of VB1a could be unequivocally confirmed using an available reference standard, corresponding to O-desmethyl venlafaxine. For this reason, a semi-quantitative evaluation was also possible. Venlafaxine showed concentration levels ranging from 0.1 to 3.1 ng/mL, while its main metabolite from 0.8 to 7.1 ng/mL. The later always presented higher concentration levels than venlafaxine itself. Hence, it would be recommendable to include the active O-desmethyl venlafaxine metabolite in monitoring programs, in order to gain a more realistic understanding of its impact on the water quality.

3.2. Comparison between Italian and Spanish samples

These studies allow us to make a comparison between the positive findings present in waters from Spain and from Italy. Table 3SI shows the omeprazole, venlafaxine and their metabolites found in 15 Spanish IWW samples. These results were obtained after a retrospective search in UHPLC-QTOF MS data.

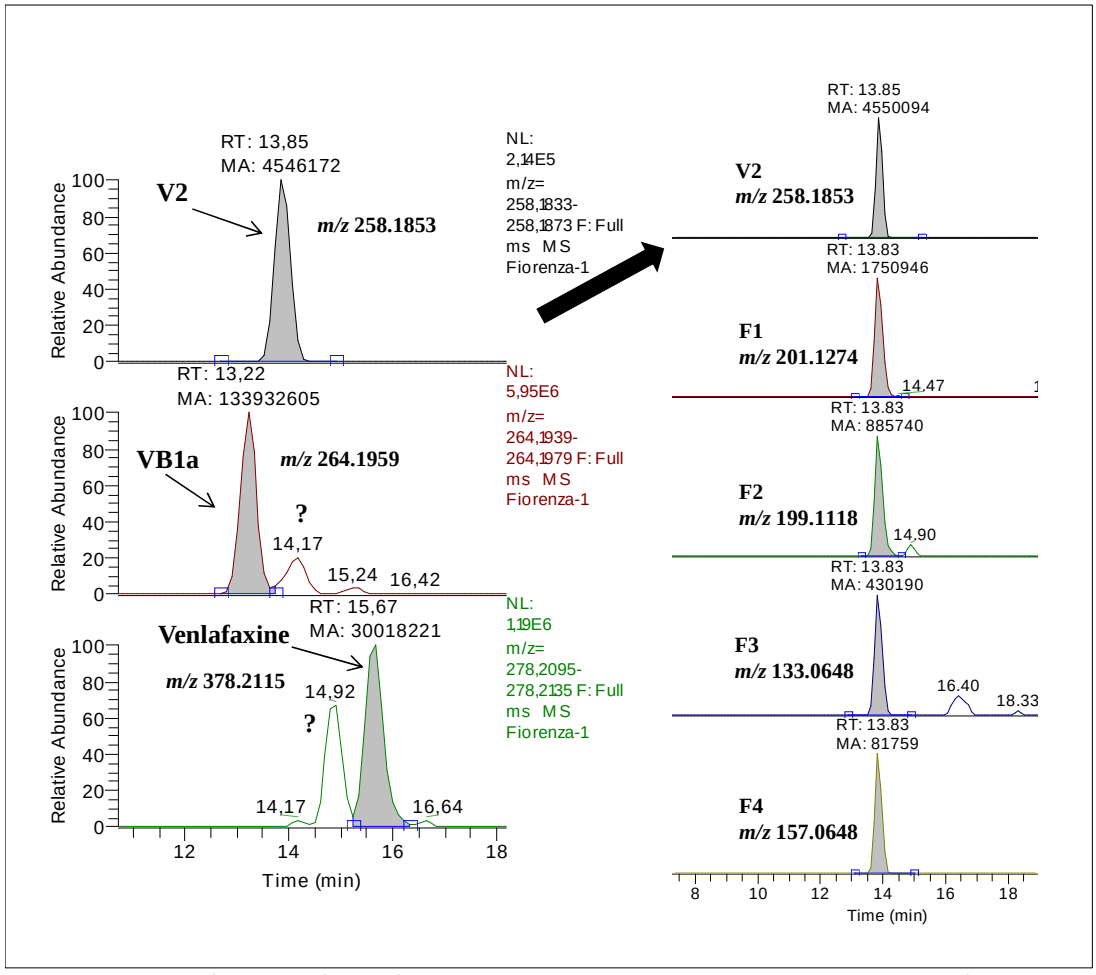

Figure 3. Positive findings of venlafaxine and its metabolites VB1a (O-desmethyl venlafaxine) and V2 in Firenze IWW sample.

On the one hand, omeprazole residues found in both countries showed results quite similar. Omeprazole was not present in any of the countries. However, 7 out of 24 OMs could be detected in Spanish IWWs (OM7c-7d, OM10, OM11a, OM14a, OM14b and OM13). It is worth to mention, that these metabolites were the same than those found in Italy

(except OM14a, which was not observed in Italy). In both cases, the compound most frequently detected was the OM10 (100% in Italian and 80% in Spanish IWWs). Then, OM7d, OM11a and OM14b were found in 20% of the Spanish samples analyzed. OM13 and OM14a were present in a 13%, and finally OM7c in a 7%.

On the other hand, venlafaxine and 3 of its metabolites could be detected in 15 Spanish IWW samples analyzed. VB1a was the compound most frequently detected (67%), followed by the parent compound (60%) and V2 (47%). The detection of these compounds was in agreement with the results obtained by Italian waters (for both 100%). Moreover, in samples from Spain, the metabolite V1 could also be detected and identified in 2 out of 15 samples.

4. CONCLUSIONS

This work reports the study of 2 pharmaceuticals (omeprazole and venlafaxine) and their metabolites in the aquatic environment. A total of 23 omeprazole and 4 venlafaxine metabolites previously studied by LC-QTOF MS working under MS^E mode (Boix et al., 2014a; Boix et al., 2014b), were considered in the present work. 30 influent wastewater samples, previously analyzed by LC-Orbitrap MS, were retrospectively processed, showing the presence of 6 omeprazole metabolites. On the contrary, omeprazole parent was not present in any of the samples. For this reason, monitoring the presence of omeprazole in aquatic environment should be focused on the omeprazole metabolites instead of the parent compound. Regarding venlafaxine, this pharmaceutical and 2 of its metabolites were found in all of the samples analyzed. It is important to appoint that in some cases its metabolites showed more abundance than the corresponding parent compound. For example, O-desmethyl venlafaxine (VB1a) presented higher estimated concentration levels (0.8-7.1 ng/mL) than venlafaxine (0.1-3.1 ng/mL). Consequently, it is suggested that future monitoring programs for water analysis could also include the O-desmethyl venlafaxine besides the parent compound. Some of the metabolites reported in this article would need to be investigated in more detail to have a more realistic overview on pharmaceutical impact in the aquatic environment. Reference standards would be required to unequivocally confirm the identity of these compounds, and to develop analytical methodologies able to accurately quantify their concentration levels in waters.

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REFERENCES

Baker, D.R., Barron, L., Kasprzyk-Hordern, B., 2014. Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates. Sci. Total Environ. 487, 629–41.

Baker, D.R., Kasprzyk-Hordern, B., 2011. Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography-mass spectrometry. J. Chromatogr. A 1218, 8036–8059.

Baker, D.R., Kasprzyk-Hordern, B., 2013. Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: new developments. Sci. Total Environ. 454-455, 442–56.

Besse, J.P., Latour, J.F., Garric, J., 2012. Anticancer drugs in surface waters. What can we say about the occurrence and environmental significance of cytotoxic, cytostatic and endocrine therapy drugs? Environ. Int. 39, 73–86.

Bijlsma, L., Boix, C., Niessen, W.M.A., Ibáñez, M., Sancho, J.V., Hernández, F., 2013. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. Sci. Total Environ. 443, 200–208.

Boix, C., Ibáñez, M., Sancho, J.V., Niessen, W.M.A., Hernández, F., 2013. Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry: degradation experiments. J. Mass Spectrom. 48, 1091–100.

Boix, C., Ibáñez, M., Zamora, T., Sancho, J.V., Niessen, W.M.A., Hernández, F., 2014a. Identification of new omeprazole metabolites in wastewaters and surface waters. Sci. Total Environ. 468, 706–14.

Boix, C., Ibáñez, M., Sancho, J.V., Parsons, J.R., de Voogt, P., Hernández, F., 2014b. Biodegradation of pharmaceuticals during waste water treatment and in surface water: identification and occurrence of transformation products. Submitted in a Journal.

Boix, C., Ibáñez, M., Sancho, J.V., Rambla, J., Aranda, J.L., Ballester, S., Hernández, F., 2015. Fast determination of 40 drugs in water using direct sample injection liquid chromatography-tandem mass spectrometry. Talanta 131, 719-727.

Boleda, M.R., Galceran, M.T., Ventura, F., 2013. Validation and uncertainty estimation of a multiresidue method for pharmaceuticals in surface and treated waters by liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1286, 146–58.

Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R., Fanelli, R., 2003. Strategic Survey of Therapeutic Drugs in the Rivers Po and Lambro in Northern Italy. Environ. Sci. Technol. 37, 1241–1248.

Castiglioni, S., Bagnati, R., Calamari, D., Fanelli, R., Zuccato, E., 2005. A multiresidue analytical method using solid-phase extraction and high-pressure liquid chromatography tandem mass spectrometry to measure pharmaceuticals of different therapeutic classes in urban wastewaters. J. Chromatogr. A 1092, 206–15.

Castiglioni, S., Bagnati, R., Fanelli, R., Pomati, F., Calamari, D., Zuccato, E., 2006. Removal of Pharmaceuticals in Sewage Treatment Plants in Italy. Environ. Sci. Technol. 40, 357–363.

Center for Promotion of Imports from Developing Countries (CBI). Pharmaceutical products: the EU market for synthetic generic medicines. Available fromhttp://www.cbi.eu/system/files/marketintel/200920-20synthetic20generic20medicines1.pdf, 2009. [Accessed on July 2013].

Chitescu, C.L., Oosterink, E., de Jong, J., Linda Stolker, A.A.M., 2012. Accurate mass screening of pharmaceuticals and fungicides in water by U-HPLC-Exactive Orbitrap MS. Anal. Bioanal. Chem. 403, 2997–3011.

Díaz, R., Ibáñez, M., Sancho, J.V., Hernández, F., 2013. Qualitative validation of a liquid chromatography-quadrupole-time of flight mass spectrometry screening method for organic pollutants in waters. J. Chromatogr. A 1276, 47–57.

Dorival-García, N., Zafra-Gómez, A., Cantarero, S., Navalón, A., Vílchez, J.L., 2013. Simultaneous determination of 13 quinolone antibiotic derivatives in wastewater samples using solid-phase extraction and ultra performance liquid chromatography–tandem mass spectrometry. Microchem. J. 106, 323–333.

Ferrer, I., Thurman, E.M., 2012. Analysis of 100 pharmaceuticals and their degradates in water samples by liquid chromatography/quadrupole time-of-flight mass spectrometry. J. Chromatogr. A 1259, 148–57.

Gasser, G., Pankratov, I., Elhanany, S., Werner, P., Gun, J., Gelman, F., Lev, O., 2012. Field and laboratory studies of the fate and enantiomeric enrichment of venlafaxine and O-desmethylvenlafaxine under aerobic and anaerobic conditions. Chemosphere 88, 98–105.

González Alonso, S., Catalá, M., Maroto, R.R., Gil, J.L.R., de Miguel, Á.G., Valcárcel, Y., 2010. Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain). Environ. Int. 36, 195–201.

Gracia-Lor, E., Ibáñez, M., Zamora, T., Sancho, J.V., Hernández, F., 2014. Investigation of pharmaceutical metabolites in environmental waters by LC-MS/MS. Environ. Sci. Pollut. Res. Int. 21, 5496–510.

Gracia-Lor, E., Sancho, J.V., Hernández, F., 2011. Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1218, 2264–2275.

Gros, M., Rodríguez-Mozaz, S., Barceló, D., 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem. J. Chromatogr. A 1248, 104–21.

Hernández, F., Ibáñez, M., Gracia-Lor, E., Sancho, J.V., 2011. Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater. J. Sep. Sci. 34, 3517–3526.

Hernando, M.D., Gómez, M.J., Agüera, A., Fernández-Alba, A.R., 2007. LC-MS analysis of basic pharmaceuticals (beta-blockers and anti-ulcer agents) in wastewater and surface water. Pharm. Anal. 26, 581–594.

Howell, S.R., Husbands, G.E., Scatina, J.A., Sisenwine, S.F., 1993. Metabolic disposition of 14C-venlafaxine in mouse, rat, dog, rhesus monkey and man. Xenobiotica. 23, 349–59.

Huntscha, S., Singer, H.P., McArdell, C.S., Frank, C.E., Hollender, J., 2012. Multiresidue analysis of 88 polar organic micropollutants in ground, surface and wastewater using online mixed-bed multilayer solid-phase extraction coupled to high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1268, 74–83.

NDC. The internet drug index (the top 200 prescriptions for 2011 by number dispensed), 2011. http://www.rxlist.com. Accessed June 2014.

Ortiz de García, S., Pinto Pinto, G., García Encina, P., Irusta Mata, R., 2013. Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. Sci. Total Environ. 444, 451–465.

Rúa-Gómez, P.C., Püttmann, W., 2012. Impact of wastewater treatment plant discharge of lidocaine, tramadol, venlafaxine and their metabolites on the quality of surface waters and groundwater. J. Environ. Monit. 14, 1391–9.

Senta, I., Terzic, S., Ahel, M., 2013. Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment. Water Res. 47, 705–714.

Sousa, M.A., Gonçalves, C., Cunha, E., Hajšlová, J., Alpendurada, M.F., 2011. Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS. Anal. Bioanal. Chem. 399, 807–822.

Stuart, M., Lapworth, D., Crane, E., Hart, A., 2012. Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1–21.

Valcárcel, Y., González Alonso, S., Rodríguez-Gil, J.L., Gil, A., Catalá, M., 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84, 1336–1348.

Van Nuijs, A.L.N., Tarcomnicu, I., Simons, W., Bervoets, L., Blust, R., Jorens, P.G., Neels, H., Covaci, A., 2010. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater. Anal. Bioanal. Chem. 398, 2211–2222.

Wode, F., Reilich, C., van Baar, P., Dünnbier, U., Jekel, M., Reemtsma, T., 2012. Multiresidue analytical method for the simultaneous determination of 72 micropollutants in aqueous samples with ultra high performance liquid chromatography-high resolution mass spectrometry. J. Chromatogr. A 1270, 118–26.

Writer, J.H., Ferrer, I., Barber, L.B., Thurman, E.M., 2013. Widespread occurrence of neuro-active pharmaceuticals and metabolites in 24 Minnesota rivers and wastewaters. Sci. Total Environ. 461-462, 519–27.

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., 2000. Presence of therapeutic drugs in the environment. Lancet 355, 1789–1790.

Zuccato, E., Castiglioni, S., Fanelli, R., 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. J. Hazard. Mater. 122, 205–209.

SUPPLEMENTARY INFORMATION

Investigation of omeprazole and venlafaxine metabolites in wastewater making use of high resolution mass spectrometry

Table 1SI. Elemental composition, exact mass and main fragment ions, obtained by LC-ESI-QTOF MS, of omeprazole, venlafaxine and their metabolites.

Compound	Elem Comp	Exact mass	Fragment ions			
Omeprazole	$C_{17}H_{20}N_3O_3S$	346.1220	198.0584	180.0478	151.0992	150.0914
OM1	$C_{20}H_{25}N_4O_5S_2$	465.1261	344.1064	313.1421	298.1187	284.1394
OM2a	$C_{23}H_{28}N_3O_9S$	522.1541	346.1220	313.1421	298.1187	182.0635
OM2b	$C_{23}H_{28}N_3O_9S$	522.1541	489.1742	346.1220	328.1115	313.1421
OM2c	$C_{23}H_{28}N_3O_9S$	522.1541	346.1220	328.1115	313.1421	298.1187
OM3	$C_{16}H_{17}N_3O_4S$	348.1013	214.0533	196.0427	152.0707	135.0553
OM4	$C_{16}H_{18}N_3O_3S$	332.1064	299.1265	284.1030	198.0584	152.0707
OM5	$C_{16}H_{18}N_3O_5S_2$	396.0683	364.0962	316.1115	283.1316	268.1081
OM6	$C_{17}H_{17}N_3O_4$	328.1292	195.0765	120.0808		
OM7a	$C_{17}H_{20}N_3O_5S$	378.1119	230.0482	212.0376	168.0656	149.0710
OM7b	$C_{17}H_{20}N_3O_5S$	378.1119	230.0482	166.0863	149.0710	138.0914
OM7c	$C_{17}H_{20}N_3O_5S$	378.1119	314.1500	230.0482	195.0223	180.0656
OM7d	$C_{17}H_{20}N_3O_5S$	378.1119	360.1013	296.1394	280.1081	230.0482
OM7e	$C_{17}H_{20}N_3O_5S$	378.1119	360.1013	230.0482	166.0863	149.0710
OM8a	$C_{17}H_{20}N_3O_4S$	362.1170	344.1064	329.1371	314.1136	310.1187
OM8b	$C_{17}H_{20}N_3O_4S$	362.1170	344.1064	329.1371	214.0533	196.0427
OM8c	$C_{17}H_{20}N_3O_4S$	362.1170	214.0533	184.0969	179.0274	166.0863
OM9	$C_{17}H_{20}N_3O_6S$	394.1068	376.0962	312.1337	296.1030	195.0223
OM10	$C_{17}H_{18}N_3O_4S$	360.1013	327.1214	312.0979	149.0710	180.0656
OM11a	$C_{17}H_{20}N_3O_3S$	346.1220	313.1421	298.1187	198.0584	149.0710
OM11b	$C_{17}H_{20}N_3O_3S$	346.1220	313.1421	298.1187	198.0584	182.0635
OM13	$C_{16}H_{16}N_3O_4S$	346.0857	313.1058	298.1187	212.0376	135.0553
OM14a	$C_{16}H_{18}N_3O_2S$	316.1215	283.1316	268.1081	182.0635	150.0914

Table 1SI (Cont.). Elemental composition, exact mass and main fragment ions, obtained by LC-ESI-QTOF MS, of omeprazole, venlafaxine and their metabolites.

Compound	Elem Comp	Exact mass	Fragment ions			
Venlafaxine	$C_{17}H_{28}NO_2$	278.2115	260.2009	215.1431	121.0648	
VB1a (O-Desmethyl venla.	$C_{16}H_{26}NO_2$	264.1959	107.0492	159.0805	58.0652	81.0699
VB1b (N-Desmethyl venla.)	$C_{16}H_{26}NO_2$	264.1959	121.0648	147.0805	246.1853	159.0805
V1	$C_{16}H_{26}NO_2$	264.1959	58.0652	246.1853	139.0754	93.0699
V2	$C_{17}H_{24}NO$	258.1853	201.1274	199.1118	133.0648	157.0648

Table 2SI. Retention times (min) of omeprazole, venlafaxine and their metabolites obtained by LC-QTOF MS and LC-Orbitrap.

	Retention time (min)	
Compound	HPLC-Orbitrap	UHPLC-QTOF MS
Omeprazole	14.7	6.0
OM7a	-	4.4
OM7b	-	4.9
OM7c	14.6	5.2
OM7d	15.1	5.3
OM7e	-	5.5
OM10	13.2	4.8
OM11a	14.0	4.9
OM11b	-	4.5
OM13	11.3	3.4
OM14a	-	4.5
OM14b	14.0	5.0
Venlafaxine	15.6	5.2
VB1a	13.2	3.8
VB1b	-	5.9
V1	-	4.1
V2	13.9	4.0

Table 3SI. Positives findings of omeprazole and venlafaxine residues detected in Spanish IWW by LC-QTOF MS.

% positive findings (retrospective)	
Compound	IWW (n=15)
Omeprazole	-
OM7c	7
OM7d	20
OM10	80
OM11a	20
OM13	13
OM14a	13
OM14b (4-OH omeprazole sulfide)	20
Compound	IWW (n=15)
Venlafaxine	60
VB1a (O-Desmethyl venlafaxine)	67
VB1b (N-Desmethyl venlafaxine)	-
V1	13
V2	47

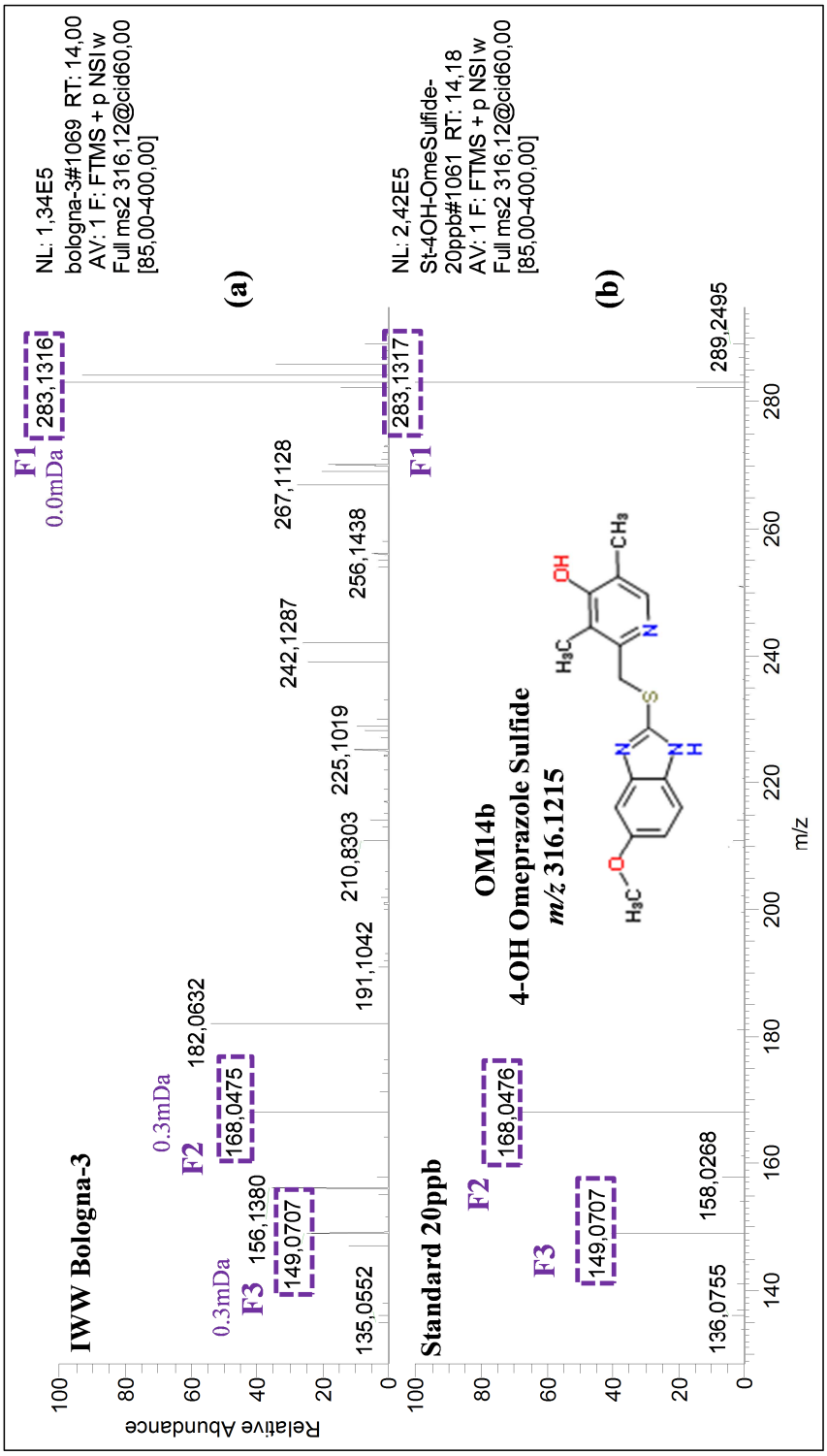

Figure 1SI. MS/MS spectra of 4-OH omeprazole sulfide in (a) Bologna IWW sample and (b) reference standard.

Table 4SI**.** Absolute areas and % of positive findings of omeprazole and venlafaxine detected in Italian IWW by LC-Orbitrap.

		Omeprazole metabolites						Venlafaxine metabolites		
		OM7c	OM7d	OM10	OM11a	OM13	OM14b	Venl	VB1a	V2
Bologna 1	Absolute area	6850111	11766702	19807465	-	4641123	7556774	100799241	237966359	13179846
	%	14	23	39	-	9	15	29	68	4
Bologna 2	Absolute area	8264045	14643530	21210574	-	3611005	8300924	110970158	239710229	19839600
	%	15	26	38	-	6	15	30	65	5
Bologna 3	Absolute area	4696781	3377566	8510975	-	1278268	-	42456996	104414391	4002808
	%	26	19	48	-	7	-	28	69	3
Milano 1	Absolute area	5214404	-	17636518	3256842	3297036	4602318	31881896	104446529	4912486
	%	15	-	52	10	10	14	23	74	3
Milano 2	Absolute area	3912436	-	9194713	1871545	2514386	4189554	28970941	61580070	3431788
	%	18	-	42	9	12	19	31	66	4
Milano 3	Absolute area	5574148	10191907	16406122	4175184	-	5737948	33267353	85634753	3268324
	%	13	24	39	10	-	14	27	70	3
Torino 1	Absolute area	7834611	12273888	26159319	3238745	-	5310596	40956036	105445541	6916023
	%	14	22	48	6	-	10	27	69	5
Torino 2	Absolute area	9083844	16041001	22309782	3243232	1400710	7739689	37790137	108586213	7469970
	%	15	27	37	5	2	13	25	71	5
Torino 3	Absolute area	8289154	10273848*	18746375	2570254	1674761	8398628	43855224	89194470	4651050
	%	17	21*	38	5	3	17	32	65	3
Merano 1	Absolute area	4185229	-	5172467	-	-	-	36847251	80832118	8583952
	%	45	-	55	-	--	-	29	64	7
Merano 2	Absolute area	3383506	-	6004013	-	886717	-	24014839	82859942	7424916
	%	33	-	58	-	9	-	21	72	6
Merano 3	Absolute area	7433679	-	5199145	-	835248	-	27667519	119090276	6771400
	%	55	-	39	-	6	-	18	78	4

Table 4SI (Cont.). Absolute areas and % of positive findings of omeprazole and venlafaxine detected in Italian IWW by LC-Orbitrap.

		Omeprazole metabolites						Venlafaxine metabolites		
		OM7c	OM7d	OM10	OM11a	OM13	OM14b	Venl	VB1a	V2
Napoli 1	Absolute area	-	-	8623137	-	-	1063325	36084656	46771345	3441968
	%	-	-	89	-	-	11	42	54	4
Napoli 2	Absolute area	1652828	2799248*	9795264	1179120	768295	3913070	29907435	25269111	3578163
	%	8	14*	49	6	4	19	51	43	6
Napoli 3	Absolute area	11658518	12704995	33782046	5038409	6547426	9174219	15610919	47342051	1617517
	%	15	16	43	6	8	12	24	73	3
Palermo 1	Absolute area	2065046	2345402	7884987	-	-	-	29769401	57833600	4641314
	%	17	19	64	-	-	-	32	63	5
Palermo 2	Absolute area	6333762	22219535	17965885	-	-	-	25152209	60497030	4989223
	%	14	48	39	-	-	-	28	67	6
Palermo 3	Absolute area	4802917	13688135	15412937	-	1097879	-	29585375	73012387	5742179
	%	14	39	44	-	3	-	27	67	5
Bari 1	Absolute area	8758130	9729125	20058968	3172055	2344014	-	51659832	103255388	2572950
	%	20	22	46	7	5	-	33	66	2
Bari 2	Absolute area	8703018	8279667	18820444	8204953	1813010	9629795	51795659	83318650	4904530
	%	16	15	34	15	3	17	37	60	4
Bari 3	Absolute area	7298329	6954094	1718028	4633182	1084374	4913686	49559237	101349997	4104965
	%	27	26	6	17	4	18	32	65	3
Firenze 1	Absolute area	1370550	1768738*	7005338	1324692	786658	-	26556010	131361442	4561722
	%	11	14*	57	11	6	-	16	81	3
Firenze 2	Absolute area	935275	3925011	6921108	1202938	-	-	20339554	105039557	2623750
	%	7	30	53	9	-	-	16	82	2
Firenze 3	Absolute area	1440627	2591924	5110055	-	608021	-	33124274	124690630	3876305
	%	15	27	52	-	6	-	20	77	2

Table 4SI (Cont.). Absolute areas and % of positive findings of omeprazole and venlafaxine detected in Italian IWW by LC-Orbitrap.

		Omeprazole metabolites						Venlafaxine metabolites		
		OM7c	OM7d	OM10	OM11a	OM13	OM14b	Venl	VB1a	V2
Verona 1	Absolute area	8336161	8958716	16579034	-	948686	4439401	3445699	112558141	3594377
	%	21	23	42	-	2	11	3	94	3
Verona 2	Absolute area	4869179	12904148	8276092	-	1098333	5381131	4892838	112164370	5849152
	%	15	40	25	-	3	17	4	91	5
Verona 3	Absolute area	5230296	8730814	13075707	-	1118254	-	5991303	91883374	3601518
	%	19	31	46	-	4	-	6	91	4
Roma 1	Absolute area	3000277	9164546	22974550	2448242	975132	3181849	31086581	83647146	5259362
	%	7	22	55	6	2	8	26	70	4
Roma 2	Absolute area	2529949	6977769	15432373	1491677	657311	2742202	25735967	76123409	5212943
	%	8	23	52	5	2	9	24	71	5
Roma 3	Absolute area	1726786	9209149	20033826	314972	1940868	2343087	24068624	76098366	5311303
	%	5	26	56	1	5	7	23	72	5

*Detected, no confirmed (no considered in Table 1).

3.5 Discusión de los resultados

La metodología de trabajo para detectar e identificar **productos de transformación** en las experiencias de laboratorio se muestra de forma esquematizada en la **Figura 3.3**.

Figura 3.3 Metodología general para la identificación de TPs.

Tras los experimentos de degradación llevados a cabo en condiciones controladas en el laboratorio, las muestras se analizaron mediante UHPLC-(Q)TOF MS, adquiriendo en modo MS^E. Los datos se procesaron con el software específico de *Waters Corp.*, MetaboLynx XS™. Esta aplicación compara los XICs de una muestra control "blanco" con otra fortificada y sometida a ensayos de degradación, de manera que los picos que aparecen en la muestra y no en el blanco son atribuidos a posibles TPs formados. En la *Figura 2* del *Artículo Científico 2*, se muestra un ejemplo de la detección del OTP 6 mediante la aplicación de dicho software.

De este modo, se detectaron hasta 17 productos de transformación del omeprazol, 5 resultaron de la hidrólisis, 5 de foto-degradación y 7 de cloración.

Todos ellos se identificaron asignándoles una fórmula molecular y un tiempo de retención (*Retention time*, Rt). Además, se intentaron elucidar aprovechando los datos adquiridos en MS^E. Generalmente, los espectros adquiridos a alta energía proporcionan una información mucho más rica, pues se favorece la fragmentación de las moléculas. La *Figura 4, Artículo Científico 2*, muestra un ejemplo de elucidación del OTP 5.

Una vez identificados de modo tentativo los 17 productos de transformación encontrados en los ensayos de laboratorio (*Figuras 3* y *5, Artículo Científico 2*), el siguiente paso fue investigar su presencia en aguas residuales y medioambientales.

Para ello, se llevó a cabo un análisis retrospectivo haciendo uso de otro software específico de *Waters Corp.*, ChromaLynx XS™, que permite la búsqueda de los compuestos incluidos en una base de datos experimental, en base a su *m*/*z* exacta teórica y a su tiempo de retención. En aquellos casos en los que se observa un pico cromatográfico (extraído con una nw-XIC de 0.02 Da), se evalúa el error de masa, el Rt observado, los iones fragmentos y el perfil isotópico. Tras el análisis de 27 aguas superficiales y 25 residuales, no se detectó omeprazol en ninguna de las muestras. Por lo que se refiere a los productos de transformación, se detectaron 2 TPs (OTP 4 y 5) en aguas residuales. Posteriormente, con el fin de confirmar la presencia de los citados positivos y de cualquier otro producto de transformación que pudiera no haber sido detectado por QTOF, se analizaron las mismas muestras de agua mediante LC-MS/MS usando un analizador más sensible como es el triple cuadrupolo. Se creó un método de SRM seleccionando los iones precursores y productos a partir de la información obtenida en los espectros de masas de alta energía del QTOF. Las energías de cono y de colisión no se pudieron optimizar, ya que no se disponía de patrones de referencia, utilizándose para todos los

compuestos valores típicos de 30 V y 25 eV, respectivamente. Tras analizar las muestras se confirmó la presencia de los OTP 4 y 5 (previamente detectados mediante el análisis retrospectivo), siendo el OTP 5 detectado en un mayor número de muestras (90% EWW y 26% SW). Adicionalmente, se encontraron los OTP 1 y OTP 13. Para más detalles ver *Artículo Científico 2.*

A pesar de que no se pudo llevar a cabo una cuantificación, al no disponer de patrones de referencia, se observó que las señales para los OTPs detectados eran bastante bajas, lo que hacía suponer que sus niveles de concentración no eran muy relevantes. Teniendo en cuenta el elevado consumo de omeprazol por la población, se esperaba encontrar mayores concentraciones de OTPs en las aguas. Por ello, se planteó una segunda hipótesis basada en una elevada metabolización del fármaco en el cuerpo humano como la principal causa de la no excreción de omeprazol como tal. De este modo, la búsqueda de residuos de omeprazol se centró en sus metabolitos. Este trabajo está descrito en el *Artículo Científico 3*.

Para realizar el estudio de **metabolitos de omeprazol**, se analizaron las orinas de tres voluntarios (2 Europeos y 1 Latino-Americana) tras la ingestión de una dosis típica de 40 mg de omeprazol. Se tomó una muestra de orina previa a la administración del fármaco y 7 muestras más durante las 24 horas posteriores a la ingesta. Una vez recogidas todas las orinas, se analizaron mediante UHPLC-QTOF MS, adquiriendo en modo MS^E. Tras el análisis y el procesamiento de los datos, se detectaron hasta 24 metabolitos comunes en las orinas de los tres voluntarios. Cabe decir, que la abundancia de ellos variaba dependiendo de cada individuo. En la **Figura 3.4** se observa el perfil de excreción de los OMs (*Omeprazole Metabolites*) más abundantes, OM 8a, 10 y 11a, en las orinas colectadas. Los voluntarios Europeos "a"

y "b" presentan un metabolismo más rápido (5-6 horas) que la Latino-Americana "c" (10-15 horas).

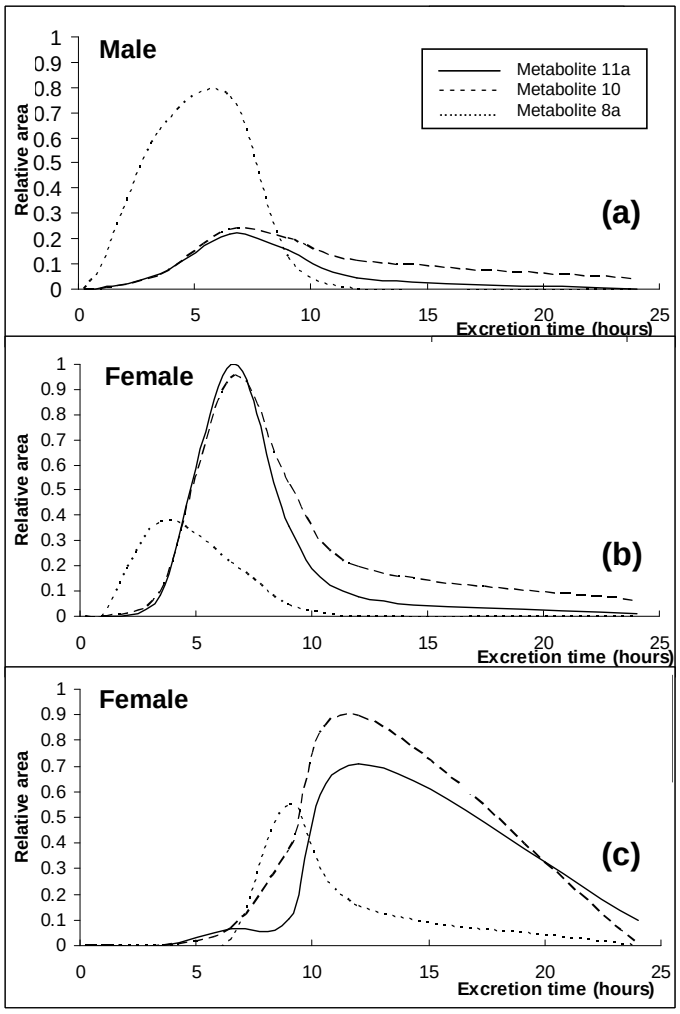

Figura 3.4 Perfil de excreción de los metabolitos del omeprazol OM 8a, 10 y 11a en orinas de (a) varón Europeo, (b) mujer Europea y (c) mujer Latino-Americana.

En términos de área absoluta, el compuesto que presentó mayor abundancia fue OM 11a (área max. ~12,000 a.u.), seguido por OM 10 (área max. ~10,000 a.u.). La mayor concentración de metabolito encontrada fue después de 6.5 horas para los voluntarios "a" y "b", y después de 12 horas para el voluntario "c".

El metabolito OM 11a se caracteriza por ser un isómero del omeprazol, es decir, comparten la misma fórmula empírica $[C_{17}H_{20}N_3O_3S]^+$. Además, en este caso particular, ambos comparten dos importantes iones fragmento (*m*/*z* 149.0715 y 198.0589). Este hecho nos llevó a considerar de manera especial dicho metabolito, ya que podría provocar falsos positivos de omeprazol en aquellos casos en los que no se lleve a cabo una separación cromatográfica adecuada.

Tras una elucidación y caracterización tentativa de los 24 metabolitos (*Figura 2, Artículo Científico 3*), se llevó a cabo un segundo análisis retrospectivo. Se realizó una evaluación retrospectiva de los datos en las mismas 52 muestras de agua que en el *Artículo Científico 2*, pero ahora con el objetivo de hallar metabolitos del omeprazol. Tras este análisis, se detectaron 9 OMs en las distintas muestras, siendo OM 10 el compuesto más abundante con una frecuencia de detección del 84% en aguas residuales (WW) y 41% en aguas superficiales (SW).

Al igual que para el estudio de TPs, se re-analizaron las muestras de agua mediante espectrometría de masas en tándem (QqQ). Se detectaron 14 de los 24 metabolitos buscados. En ninguna muestra se detectó el omeprazol, pero si su metabolito isomérico OM 11a (80% IWW, 90% EWW y 30% SW). Esto hubiera podido dar falsos positivos en el caso de desconocer su existencia. Los metabolitos OM 10, 14a y 14b fueron los compuestos más frecuentemente detectados, mostrando su presencia en todas las WW analizadas. En el caso del OM 14b su identidad pudo ser confirmada con el patrón de referencia 4-hidroxi omeprazol sulfide, al estar éste disponible (*Figura S2, Artículo Científico 3*). Para más detalles ver *Artículo Científico 3.*

Los resultados obtenidos en esta investigación y resumidos en los *Artículos Científicos 2* y *3* demuestran la importancia de llevar a cabo este tipo de trabajos para identificar correctamente los biomarcadores del uso de fármacos, de modo que se

pueda evaluar de forma correcta y realista su posible impacto sobre el medio ambiente. Frente a la no detección del fármaco omeprazol en ninguna de las muestras analizadas, se demostró que prácticamente el 100% de las muestras analizadas contenían alguno de sus metabolitos o TPs.

Finalmente, en el trabajo mostrado en el *Artículo Científico 4* se investigó la presencia de 23 metabolitos del omeprazol y 4 de la venlafaxina en 30 muestras de influente urbano procedentes de diez ciudades diferentes de Italia (Roma, Milán, Florencia, Palermo, Bari, Torino, Bolonia, Verona, Merano y Nápoles). La metodología analítica se basaba en una extracción de la muestra por SPE, seguida del análisis mediante HPLC-HRMS usando un analizador Orbitrap. Para el procesamiento de los datos se utilizó el software específico Xcalibur™ de *Thermo Scientific*.

Todas las muestras se inyectaron dos veces en el sistema LC-HRMS. En la primera inyección se adquirió en modo MS, con el objetivo de **detectar** la posible presencia de metabolitos. Aprovechando la adquisición en *full-scan* se llevó a cabo un análisis retrospectivo en los 30 influentes. Se detectó, inicialmente, la posible presencia de 10 metabolitos del omeprazol y 4 de la venlafaxina, basándose únicamente en la masa exacta de la molécula protonada.

Para la confirmación de los positivos se precisó de información de iones fragmento. Para ello se analizaron, nuevamente, los influentes mediante LC-Orbitrap, ahora con una adquisición en modo MS y MS/MS secuencial.

Los requisitos para confirmar un positivo fueron:

- Presencia de la molécula protonada $[M+H]^+$ (errores de masa < 5 ppm).
- Presencia de al menos 2 iones fragmento (errores de masa < 5 ppm).
- La elución al mismo tiempo de retención de una muestra respecto a las otras (tolerancia Rt del 2.5%).

La *Tabla 1* del *Artículo Científico 4* muestra los metabolitos del omeprazol y de la venlafaxina detectados y tentativamente identificados en las diez ciudades italianas estudiadas en el presente trabajo. Roma presentó el mayor número de positivos encontrados, con 100% para OMs y 100% para VMs.

Por lo que se refiere al omeprazol, cabe destacar, que dicho fármaco tampoco se detectó en ninguna de las muestras de influente analizadas, lo que está en concordancia con los resultados previamente obtenidos en nuestro laboratorio y reflejados en los *Artículos Científicos 2* y *3*. Además, el metabolito OM 10, fue el compuesto presente en un mayor número de muestras (100%); hecho que también coincide con los datos reportados en el *Artículo Científico 3.* Asimismo, es importante mencionar que de los 23 metabolitos de omeprazol buscados, se pudieron identificar 6 de ellos *(*OM 7c-d, 10, 11a, 13 y 14b*)* en las muestras de influente italianas. Estos compuestos coinciden con los detectados en las aguas españolas (excepto OM 14a presente únicamente en las aguas analizadas en España).

La disponibilidad del patrón de referencia 4-hidroxi omeprazol sulfide, permitió, además de la inequívoca confirmación del metabolito OM 14b, una estimación semi-cuantitativa de las concentraciones presentes en las muestras (entre 0.1-1.0 ng/mL).

En cuanto a la venlafaxina, dicho fármaco y 2 de sus metabolitos (V1Ba y V2) se detectaron en todas las IWW analizadas. VB1a se pudo confirmar con un patrón de referencia, siendo éste el principal metabolito de la venlafaxina, O-desmetil venlafaxina. En este aspecto, es importante destacar que dicho metabolito mostraba, presumiblemente, concentraciones más elevadas que la propia venlafaxina (Max conc.: metabolito 7.1 ng/mL; venlafaxina 3.1 ng/mL).

El trabajo reportado en el *Artículo Científico 4* muestra la aplicabilidad de las investigaciones realizadas previamente en nuestro laboratorio. Además, la concordancia de los resultados reportados en los tres estudios mediante análisis por LC-MS/MS QqQ, LC-QTOF MS y LC-Orbitrap MS, refuerza la veracidad de las hipótesis propuestas.

CAPÍTULO 4

INVESTIGACIÓN DE LA PRESENCIA DE CANNABIS EN AGUAS: ESTUDIO DE DEGRADACIÓN DE SU PRINCIPAL METABOLITO (THC-COOH)

4.1 Introducción

4.2 Artículo Científico 5: *Investigation of cannabis biomarkers and transformation products in waters by liquid chromatography coupled to time-of-flight and triple quadrupole mass spectrometry.* Chemosphere, 99 (2014) 64-71

4.3 Discusión de los resultados

4.1 Introducción

En este trabajo se llevó a cabo un estudio de degradación del principal metabolito del cannabis, el 11-Nor-9-carboxy-Δ^9-tetrahidrocannabinol (THC-COOH) (**Figura 4.1**), comúnmente usado como biomarcador del consumo de cannabis por la población.

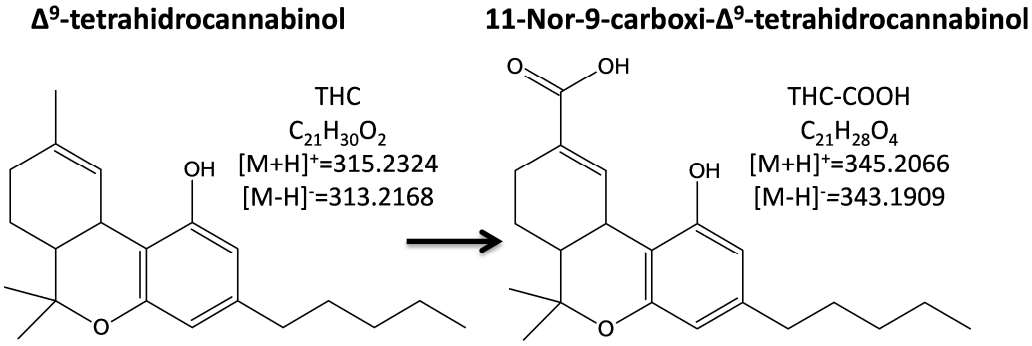

Figura 4.1 Estructuras químicas del cannabis y de su principal metabolito.

El cannabis (THC) es la droga de abuso más traficada y consumida en el mundo, tal y como se reporta en las bases de datos oficiales de las Naciones Unidas (UNODC, 2013) y de la Unión Europea (EMCDDA, 2014). En Europa, alrededor del 10% de los

jóvenes entre 15-34 años consumen esta droga, alcanzando un 20% en países como España, Francia o la República Checa (UNODC, 2006). Sin embargo, la detección del cannabis y/o su principal metabolito (THC-COOH) en las aguas resulta, en ocasiones, bastante problemática (Bijlsma, 2009; Berset, 2010). Este trabajo surgió con el objetivo de aportar más información sobre la presencia de cannabis y sus metabolitos/TPs en las aguas, intentando ayudar a solventar algunas de las dificultades encontradas en su análisis.

Se consideraron diferentes hipótesis, tanto analíticas como fisiológicas, relacionadas con la problemática detección del cannabis en aguas.

La primera hipótesis estaba relacionada con el metabolismo y grado de excreción de esta droga. El cannabis, o marihuana, es comúnmente introducido en el cuerpo humano por inhalación, absorbido a través de los pulmones y rápidamente metabolizado por el hígado (Castiglioni, 2008). Una elevada metabolización produciría la excreción del compuesto en forma de sus metabolitos mayoritarios. Se planteó que, de modo análogo al trabajo realizado en el Capítulo 3, en el que se concluye que es más realista buscar en aguas los metabolitos del omeprazol que el propio fármaco, podría ocurrir una situación semejante en el caso del cannabis y que los análisis no estuvieran dirigidos hacia el biomarcador más correcto.

La mayoría de métodos multi-residuo en los que se incluye el cannabis como compuesto a estudiar, seleccionan el THC-COOH como biomarcador (Bijlsma, 2009; Postigo, 2010; Lai, 2011; Boleda, 2009). No obstante, con esta hipótesis se pretendía comprobar si el THC-COOH era realmente el mejor metabolito, o por el contrario había otros biomarcadores más adecuados.

Hasta la fecha se habían reportado como principales metabolitos del cannabis el THC-COOH y su glucurónido. Para comprobar estos datos se analizaron, mediante UHPLC-QTOF, las orinas de tres voluntarios tras la inhalación de cannabis. Se investigaron, además del THC y su principal metabolito (de los cuales se disponía de patrón de referencia), cuatro metabolitos reportados en artículos científicos (glucurónido del THC-COOH, 11-hidroxi-Δ^9-THC, 8-hidroxi-Δ^9-THC y 8,11-dihidroxi-Δ^9-THC, de los cuales no se disponía de patrón de referencia) (Castiglioni, 2006; Fabritus, 2012; Scheidweiler, 2012). A modo de ejemplo, la **Figura 4.2** muestra los cromatogramas para el cannabis y sus metabolitos en una muestra de orina. El cannabis (THC) no se detectó en ninguna de las muestras, siendo el glucurónido del THC-COOH el principal metabolito excretado (en área absoluta), seguido del mismo THC-COOH. No se observó la presencia de los otros metabolitos reportados. Tras comprobar que el THC-COOH era el metabolito libre mayoritariamente excretado en orina, la primera hipótesis sobre la existencia de otros metabolitos relevantes se rechazó.

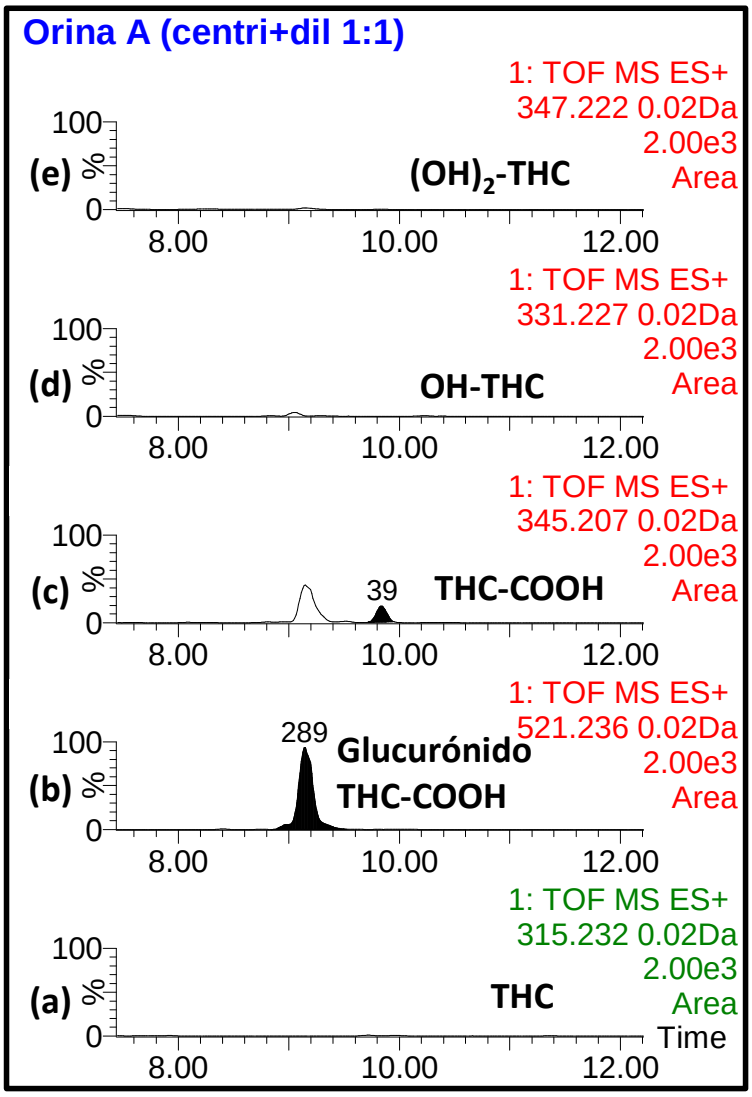

Figura 4.2 nw-XICs del (a) THC, (b) glucurónido del THC-COOH, (c) THC-COOH, (d) 11-hidroxi-Δ^9-THC y 8-hidroxi-Δ^9-THC, y (e) 8,11-dihidroxi-Δ^9-THC, en orina.

La segunda hipótesis estaba directamente relacionada con la metodología analítica utilizada. En ella se cuestionaban las condiciones utilizadas en el tratamiento de muestra, achacando la escasa presencia de cannabis en las aguas a pérdidas en el proceso de tratamiento de muestra. También podía deberse a una inadecuada optimización de los parámetros analíticos, ya sean en cuanto a la cromatografía

líquida (fase móvil, columnas cromatográficas, flujo…) como a la espectrometría de masas (energías de colisión, de la fuente de ionización, lentes…). Sobre esta hipótesis no se trabajó, pues había extensos y detallados estudios (Bijlsma, 2014; Skopp, 2004; Boleda, 2007; Berset, 2010) donde se estudiaban los parámetros óptimos para obtener la máxima sensibilidad del THC-COOH. Además, distintos trabajos reportaban buenas recuperaciones del THC-COOH en muestras de agua, utilizando su propio analito marcado como IS (Bijlsma, 2009, 2014; Castiglioni, 2008; Boleda, 2007; Berset, 2010).

A pesar de no haber trabajado sobre esta hipótesis, parece que la determinación de cannabis en aguas residuales es problemática y que hay una falta de robustez en los métodos aplicados. Actualmente, nuestro grupo, en colaboración con el *Watercyle Research Institute* (KWR) holandés, está trabajando en esta problemática analítica, por lo que cabe esperar nuevos datos en un futuro próximo.

La última hipótesis consideraba la posibilidad de una degradación/transformación de la droga (o del principal metabolito THC-COOH) tras su paso por las estaciones depuradoras de aguas residuales o en el medio ambiente posterior, cuando el compuesto llega a las aguas superficiales. Como se ha comentado en el Capítulo 1 (Introducción General), en las EDARs se llevan a cabo tres tratamientos generales, con el fin de convertir aguas brutas en aguas de una cierta calidad, que puedan ser vertidas al medio ambiente o usadas para fines agrícolas. De acuerdo con nuestros datos, no había estudios que reportaran la degradación del principal metabolito del cannabis en el medio ambiente.

Por estos motivos, se llevó a cabo el estudio presentado en el *Artículo Científico 5*, sobre degradación del THC-COOH. En él se realizaron experimentos de cloración y foto-degradación por radiación ultravioleta, tratando de simular los procesos

comúnmente aplicados en las estaciones de depuración de aguas. Por otro lado, también se realizaron experimentos de hidrólisis y de simulación de la luz solar (foto-degradación), reproduciendo, de ese modo, posibles condiciones de degradación en el medio ambiente.

Tras realizar los experimentos de degradación, las diferentes alícuotas tomadas a lo largo del tiempo se analizaron por UHPLC-ESI-QTOF MS, con el fin de detectar y elucidar posibles productos de transformación generados. En la segunda parte del trabajo se analizaron aguas residuales y superficiales, tratando de determinar la presencia de los productos de transformación previamente identificados. Estos análisis se llevaron a cabo con un analizador de triple cuadrupolo, QqQ, el cual permite detectar niveles de concentración del orden de sub-ppb, debido a su mayor sensibilidad.

El hecho de encontrar productos de transformación en las muestras de agua, evidenciaría el interés y utilidad de los experimentos a nivel de laboratorio, simulando adecuadamente las reacciones de degradación que pueden darse en el medio ambiente y/o en las estaciones depuradoras. En el apartado que sigue a continuación se presentan los datos relativos a este estudio, mostrando la detección de algunos TPs del THC-COOH en las aguas.

4.2 Artículo Científico 5

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Investigation of cannabis biomarkers and transformation products in waters by liquid chromatography coupled to time of flight and triple quadrupole mass spectrometry

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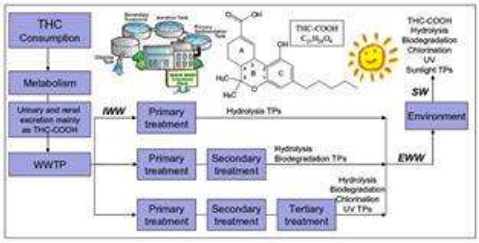
HIGHLIGHTS

- Degradation assays (hydrolysis, photo-degradation and chlorination) were performed.
- LC-QTOF MS allowed identifying up to 17 TPs under laboratory controlled conditions.
- THC-COOH was detected in 100% of wastewater and in 50% of surface water analyzed.
- 1 Hydrolysis and 5 photo-degradation TPs were detected in the water samples.
- Some of these transformation products have not been reported in the literature yet.

GRAPHICAL ABSTRACT

Schematic overview for THC-COOH presence and behavior in WWTPs and in the environment.



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ABSTRACT

11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) is commonly selected as biomarker for the investigation of cannabis consumption through wastewater analysis. The removal efficiency of THC-COOH in wastewater treatment plants (WWTPs) has been reported to vary between 31% and 98%. Accordingly, possible transformation products (TPs) of this metabolite might be formed during treatment processes or in receiving surface water under environmental conditions. In this work, surface water was spiked with THC-COOH and subjected to hydrolysis, chlorination and photo-degradation (both ultraviolet and simulated sunlight) experiments under laboratory-controlled conditions. One hydrolysis, eight chlorination, three ultraviolet photo-degradation and seven sunlight photo-degradation TPs were tentatively identified by liquid chromatography coupled to quadrupole time-of-flight mass spectrometer (LC-QTOF MS). In a subsequent step, THC-COOH and the identified TPs were searched in wastewater samples using LC coupled to tandem mass spectrometry (LC–MS/MS) with triple quadrupole. THC-COOH was found in all influent and effluent wastewater samples analyzed, although at significant lower concentrations in the effluent samples. The removal efficiency of WWTP under study was approximately 86%. Furthermore, THC-COOH was also investigated in several surface waters, and it was detected in 50% of the samples analyzed. Regarding TPs, none were found in influent wastewater, while one hydrolysis and five photo-degradation (simulated sunlight) TPs were detected in effluent and surface waters. The most detected compound, resulting from sunlight photo-degradation, was found in 60% of surface waters analyzed. This fact illustrates the importance of investigating these TPs in the aquatic environment.

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INVESTIGATION OF CANNABIS BIOMARKERS AND TRANSFORMATION PRODUCTS IN WATERS BY LIQUID CHROMATOGRAPHY COUPLED TO TIME OF FLIGHT AND TRIPLE QUADRUPOLE MASS SPECTROMETRY

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ABSTRACT

11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) is commonly selected as biomarker for the investigation of cannabis consumption through wastewater analysis. The removal efficiency of THC-COOH in wastewater treatment plants (WWTPs) has been reported to vary between 31% and 98%. Accordingly, possible transformation products (TPs) of this metabolite might be formed during treatment processes or in receiving surface water under environmental conditions. In this work, surface water was spiked with THC-COOH and subjected to hydrolysis, chlorination and photo-degradation (both ultraviolet and simulated sunlight) experiments under laboratory-controlled conditions. One hydrolysis, eight chlorination, three ultraviolet photo-degradation and seven sunlight photo-degradation TPs were tentatively identified by liquid chromatography coupled to quadrupole time-of-flight mass spectrometer (LC-QTOF MS). In a subsequent step, THC-COOH and the identified TPs were searched in wastewater samples using LC coupled to tandem mass spectrometry (LC–MS/MS) with triple quadrupole. THC-COOH was found in all influent and effluent wastewater samples analyzed, although at significant lower concentrations in the effluent samples. The removal efficiency of WWTP under study was approximately 86%. Furthermore, THC-COOH was also investigated in several surface waters, and it was detected in 50% of the samples analyzed. Regarding TPs, none were found in influent wastewater, while one hydrolysis and five photo-

degradation (simulated sunlight) TPs were detected in effluent and surface waters. The most detected compound, resulting from sunlight photo-degradation, was found in 60% of surface waters analyzed. This fact illustrates the importance of investigating these TPs in the aquatic environment.

Keywords

Cannabis biomarkers, wastewater, surface water, removal efficiency, transformation/degradation products, time-of-flight mass spectrometry.

Highlights

- Degradation assays (hydrolysis, photo-degradation and chlorination) were performed.
- LC-QTOF MS allowed identifying up to 17 TPs under laboratory controlled conditions.
- THC-COOH was detected in 100% of wastewater and in 50% of surface water analyzed.
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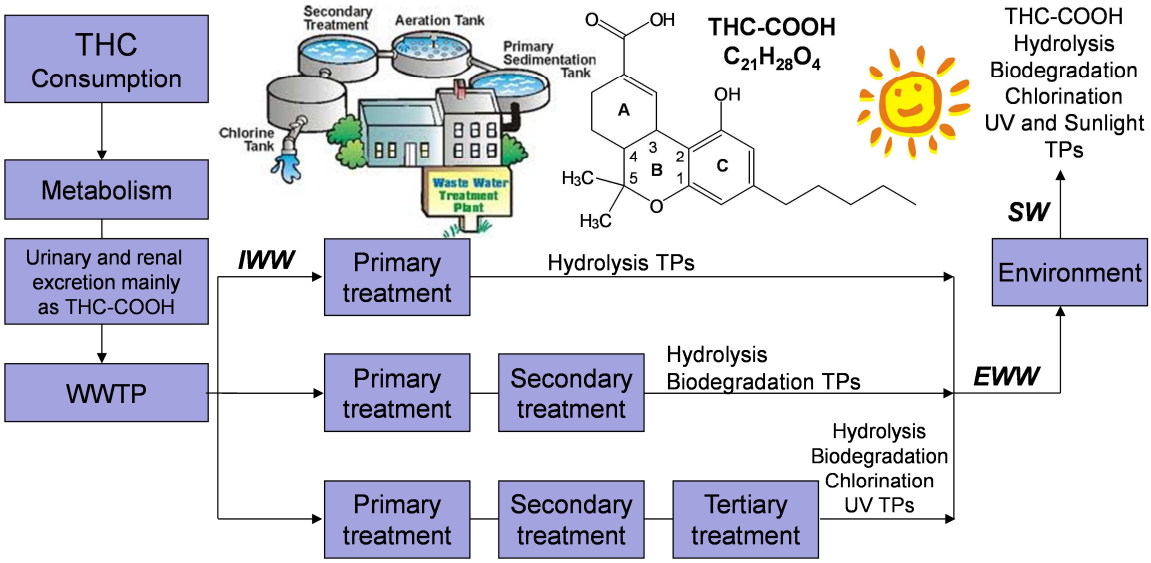


Graphical abstract. Schematic overview for THC-COOH presence and behavior in WWTPs and in the environment.

1. INTRODUCTION

Cannabis is the most widely used illicit drug in Europe (EMCDDA, 2010). Its psychoactive compound, Δ^9-tetrahydrocannabinol (THC), is extensively metabolized leading to low excretion rates as unchanged compound (Postigo et al., 2010). 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) and its glucuronide are the main metabolites of cannabis in urine (Weinmann et al., 2001 and Skopp and Pötsch, 2004). This fact has led researchers to select THC-COOH as biomarker to estimate cannabis consumption from wastewater analysis (Lai et al., 2011 and van Nuijs et al., 2011) and also in environmental studies (Bijlsma et al., 2009, Boleda et al., 2009, Berset et al., 2010 and Vazquez-Roig et al., 2010).

THC-COOH enters wastewater treatment plants (WWTPs) after the consumption of cannabis. There are several treatment processes that may be performed inside the WWTPs. While primary and secondary treatments are applied in most WWTPs, only some of them use additional processes, such as ozonation, ultraviolet light (UV) or chlorination (EPA, 2004). During these treatments, THC-COOH can be removed and/or transformed into different transformation products (TPs) that may be released in receiving surface water (SW). Therefore, the detection and confirmation of cannabinoids in aqueous samples is important from an environmental perspective (Boleda et al., 2009 and Vazquez-Roig et al., 2010).

It is common to report lower concentrations of THC-COOH in effluent wastewater (EWW) than in influent wastewater (IWW) (Castiglioni et al., 2006, Boleda et al., 2007, Bijlsma et al., 2009, Postigo et al., 2010 and Bijlsma et al., 2012). From these data, it may imply that THC-COOH is partially eliminated in WWTPs. Different percentages of THC-COOH removal efficiency have been reported in the literature, ranging between 31% and 98% (Boleda et al., 2009, Postigo et al., 2010 and Bijlsma et al., 2012). Moreover, some papers reported the detection of this metabolite in surface water at low levels (Boleda et al., 2007, Postigo et al., 2010 and Vazquez-Roig et al., 2010). It may be expected that different TPs are generated by transformation/degradation processes in WWTPs but also under

environmental conditions in the aquatic ecosystem. The ecotoxic, mutagenic and other potential effects of TPs are mostly unknown and need to be investigated (Fatta-Kassinos et al., 2011a and Fatta-Kassinos et al., 2011b). Only limited data shows that some TPs are as hazardous, or even more so, than the parent compound, producing negative effects on humans and wildlife (Farré et al., 2008, Fatta-Kassinos et al., 2011a, Fatta-Kassinos et al., 2011b, Gosetti et al., submitted for publication and Kern et al., 2009). For these reasons, it is important to investigate the possible presence of THC-COOH TPs in the environment due to the wide consumption of cannabis around the world.

The analytical determination of THC-COOH in waters is mostly based on liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS), a robust and well-established technique for the sensitive determination of illicit drugs in the aquatic environment (Castiglioni et al., 2006, Boleda et al., 2007, Bijlsma et al., 2009, Postigo et al., 2010 and Thomas et al., 2012). High resolution mass spectrometry (HRMS) instruments, such as Orbitrap (Kern et al., 2009, Wick et al., 2011 and Bijlsma et al., 2013b) and time-of-flight (TOF) (Ibáñez et al., 2004, Quintana et al., 2010, Ibáñez et al., 2011 and Bijlsma et al., 2013a), are advanced analytical tools for the tentative identification and elucidation of TPs, thanks to the sensitive accurate-mass full-spectrum acquisition provided by these analyzers. In addition, hybrid analyzers, such as (Q)TOF MS, allow data acquisition under MS^E mode (Hernández et al., 2011 and Boix et al., 2013), obtaining simultaneously the accurate masses of both (de)protonated molecules and the fragment ions in a single injection. This is highly useful for identification/elucidation purposes.

The objective of this paper is to perform an investigation on THC-COOH as cannabis biomarker in waters and on the formation of possible TPs, using LC-(Q)TOF MS under MS^E acquisition mode. For this purpose, laboratory controlled degradation experiments (hydrolysis, chlorination and photo-degradation) were first carried out trying to tentatively identify and elucidate the formed TPs using LC-(Q)TOF-MS. In a second step, THC-COOH and the TPs identified in the laboratory experiments were searched by LC-QqQ MS, in both influent and effluent wastewaters, in order to investigate the effect of the treatment processes on generating these TPs in the WWTPs. Several surface water

samples were also analyzed to know whether the THC-COOH TPs are present in the aquatic environment.

2. METHODS

2.1. Reagents and chemicals

A reference standard of THC-COOH was purchased from the National Measurement Institute (Pymble, Australia). A stock solution of 100 mg L^{-1} was prepared in methanol (MeOH). A working solution (10 mg L^{-1}) was made by ten times diluting the stock solution with MeOH.

HPLC-grade methanol (MeOH), acetronitrile (ACN), sodium hydroxide (NaOH, 99%) and formic acid (FA, 98–100%) were acquired from Scharlau (Barcelona, Spain). A Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA) was used to obtain the HPLC grade water. Leucine enkephalin, used as the lock mass (*m*/*z* 556.2771 in positive- and *m*/*z* 554.2615 in negative-ion mode) was purchased from Sigma–Aldrich.

Solid-phase extraction (SPE) cartridges (Oasis-HLB; 3 mL, 60 mg) were purchased from Waters (Milford, MA, USA).

2.2. Instrumentation

2.2.1. LC-ESI-QTOF MS

An ultra-high-performance liquid chromatography (UHPLC) system (Waters Acquity, Milford, MA, USA) was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Premier, Waters Micromass, Manchester, UK) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operating in both positive- and negative-ion modes and controlled by MassLynx v 4.1 software. The chromatographic separation was performed using an Acquity UPLC BEH C18 100 mm × 2.1 mm, 1.7 μm particle size analytical column (Waters). The mobile phases used were A = H_2O and B = MeOH, both with 0.01% FA. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10%; 9 min, 90%; 11 min, 90%; 11.1 min, 10%; 14 min, 10%. The flow rate was 0.3 mL min^{-1}. The column and sample temperatures were kept at 40 °C and 5 °C, respectively. For MS^E experiments, two acquisition functions with different collision energies were created: the low-energy (LE)

function with a collision energy of 4 eV, and the high energy (HE) function with a collision energy ramp ranging from 15 to 40 eV. The same cone voltage (15 V) and collision energy ramp was used for additional MS/MS experiments. Further details on instrument operating conditions can be found elsewhere (Boix et al., 2013).

Data were processed using MetaboLynx XS software (within MassLynx).

2.2.2. LC-ESI-QqQ MS

An ultra-high-performance liquid chromatography system (Waters Acquity, Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operating in positive (3.0 kV) and negative (−2.0 kV) ion modes. The chromatographic separation was obtained using the same analytical column and chromatographic conditions as for QTOF analysis. Cone gas as well as desolvation gas was nitrogen (Praxair, Valencia, Spain). The cone gas and the desolvation gas flows were set to 250 L h^{-1} and 1200 L h^{-1}, respectively. For operation in the MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) with a pressure of 4×10^{-3} mbar in the collision cell (0.15 mL min^{-1}). Source and desolvation temperatures were 150 °C and 650 °C, respectively. Dwell times were automatically selected.

Data were processed using TargetLynx software (within MassLynx).

2.3. Degradation experiments

Several degradation experiments were performed trying to simulate environmental conditions (hydrolysis and sunlight photo-degradation) and some processes that can occur in wastewater treatment plants (hydrolysis, chlorination and UV photo-degradation). Biodegradation experiments were not performed because no adequate material is available at our laboratory. The general strategy for identification of TPs using UHPLC-ESI-QTOF MS can be found elsewhere (Acero et al., 2008, Ibáñez et al., 2004, Ibáñez et al., 2006, Hernández et al., 2008, Trovó et al., 2009 and Bijlsma et al., 2013a).

Surface water from the Mijares River (Castellón, Spain) was collected in November 2011 and used for all laboratory controlled experiments (Table 1SI shows the main physic-chemical characteristics of the water used in laboratory experiments). The samples used for hydrolysis, chlorination and photo-degradation experiments were spiked with THC-COOH at 1 mg L^{-1} (for UV experiments 0.5 mg L^{-1}). This relative high concentration allowed better detection and evaluation of TPs, and facilitated the detection of minor TPs. Non-spiked surface water samples were subjected to the same degradation processes and used as control samples.

Hydrolysis experiments were performed in darkness at room temperature. 2-mL aliquots were sampled at different time intervals (0, 1 d, 3 d, 7 d, 10 d and 17 d) and immediately stored at −20 °C.

Chlorination experiments were performed adding 40 µL of commercial NaClO 1% w/v solution to 50 mL of spiked sample. To study degradation kinetics in time, 2-mL aliquots at different time intervals (0, 5 min, 15 min, 30 min, 45 min, 1.5 h, 3 h, 8 h, 23 h and 33 h) were sampled and stored at −20 °C (Bijlsma et al., 2013a).

Photo-degradation experiments were carried out under UV radiation and simulated sunlight. UV radiation was performed using a mercury lamp with its main output at 254 nm. The 250 mL surface water samples were kept in quartz glass vessels at a distance of ~15 cm from the lamp. The experiment was carried out in a fume hood at room temperature over a period of 72 h under constant stirring of the samples (0, 30 min, 1.5 h, 4 h, 7 h, 20 h, 30 h, 53 h and 72 h). Sunlight was simulated using a solar simulation system (Suntest XLS+, Atlas MTT, Linsengericht, Germany), equipped with a xenon arc lamp as radiation source and a solar light filter allowing a wavelength in the range of 300–800 nm. The radiation intensity was set to 500 W m^{-2} and the light dose per hour of irradiation to 1.8 MJ h^{-1}. In this way, 90 irradiation hours corresponds to 15 d of natural sun light (dose: 288 MJ m^{-2}). The degradation was performed using 250-mL closed quartz glass vessels and sample temperature was set to 25 °C in order to minimize sample evaporation and possible thermal transformation. Aliquots were sampled after stirring of the water

solution. During irradiation, 2-mL water samples were taken at different time intervals (0, 30 min, 2 h, 8 h, 20 h, 30 h, 50 h and 80 h) and immediately stored at −20 °C. In the photo-degradation experiments, evaporation of the samples was observed and assessed by correcting the peak-areas as a function of the measured volume.

2.4. Data processing

MetaboLynx XS application manager (Micromass v 4.1) was used to process QTOF MS data obtained from degradation studies. This software compares eXtracted Ion Chromatograms (XICs) of a positive/degraded sample to a control sample for detecting, identifying and reporting differential ions/chromatographic peaks which would correspond, in principle, to transformation products (Ibáñez et al., 2006 and Boix et al., 2013).

TargetLynx (also from Micromass v 4.1) was employed for automatically processing data from triple quadrupole analysis. For confirmation of positive findings, the acquisition of two selected reaction monitoring (SRM) transitions per compound together with the agreement in both retention time and Q/q ratio deviation were required (Commission Decision, 2002). Reference Q/q ratios were obtained from TPs identified in degradation experiments as the ratio between the most abundant transition (Q, quantitative) and the other/s measured transition/s (q, confirmation) (Boix et al., 2013).

2.5. Water samples

Ten 24-h composite wastewater samples (five IWW and five EWW) were collected from a WWTP located in Castellón (Eastern Spain), which main characteristics are summarized in Table 2SI. Ten grab surface samples were collected from four sites of Albufera Natural Park of Valencia (Eastern Spain) and from six sites of two Dutch rivers (Rhine and Meuse). All samples were taken from March to May in 2012, and immediately stored at −20 °C.

2.6. Sample treatment

A SPE step was applied prior analysis to pre-concentrate the sample. SPE Oasis HLB cartridges were conditioned with 3 mL MeOH and 3 mL Milli-Q water. After loading 50 mL water sample (IWW were previously 4-fold diluted with Milli-Q water), cartridges were vacuum dried for 5 min. Analytes were eluted with 5 mL MeOH. The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen and reconstructed in 1 mL of 10:90 MeOH:H_2O. UHPLC–MS/MS QqQ analyses were performed by injecting 50 µL of the final extract into the system.

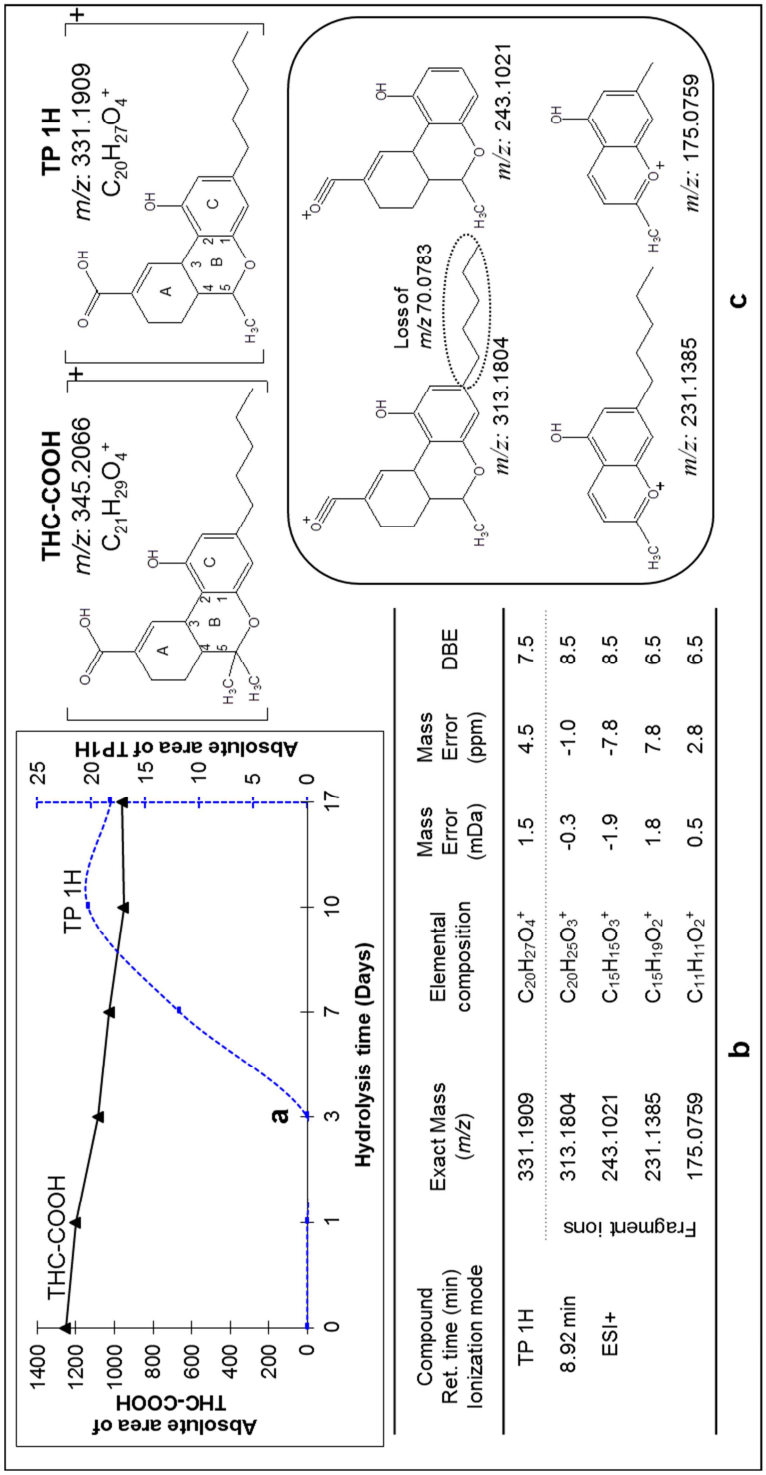
3. RESULTS AND DISCUSSION

3.1. Degradation experiments

3.1.1. Hydrolysis

Fig. 1a shows the hydrolytic degradation of THC-COOH during 17 d in darkness at room temperature. THC-COOH was transformed (around 20%) into TP 1H, which appeared on the third day reaching its maximum concentration after 10 d.

Information on elemental composition, retention time, fragment ions, mass errors and double bond equivalent (DBE) obtained for TP 1H is shown in Fig. 1b. This TP was detected in positive ionization mode, eluting at 8.92 min. Its elemental composition, $C_{20}H_{27}O_4^+$, implies the loss of one methyl group from the THC-COOH molecule. The demethylation could have occurred in two sites of the original structure: in ring B or in aliphatic chain. The loss observed at 70.0783 Da might correspond to the aliphatic chain (C_5H_{10}, +1.1 mDa). This would indicate that TP 1H structure would be more feasible if the demethylation from THC-COOH occurred in the B ring (pos. 5). A possible structure of the protonated molecule of this TP and structures of the fragment ions are shown in Fig. 1c.



Compound Ret. time (min) Ionization mode		Exact Mass (*m/z*)	Elemental composition	Mass Error (mDa)	Mass Error (ppm)	DBE
TP 1H		331.1909	$C_{20}H_{27}O_4^+$	1.5	4.5	7.5
8.92 min	Fragment ions	313.1804	$C_{20}H_{25}O_3^+$	-0.3	-1.0	8.5
ESI+		243.1021	$C_{15}H_{15}O_3^+$	-1.9	-7.8	8.5
		231.1385	$C_{15}H_{19}O_2^+$	1.8	7.8	6.5
		175.0759	$C_{11}H_{11}O_2^+$	0.5	2.8	6.5

Fig. 1. (a) Degradation of THC-COOH under hydrolysis conditions and TP formed, (b) elemental composition, retention time, fragments ions, mass errors and DBE and (c) proposed structures for TP 1H and for its fragment ions.

3.1.2. Chlorination

The study of THC-COOH TPs was performed using a chlorine concentration commonly applied in a tertiary treatment in WWTP (0.8 mg L^{-1}) (Bijlsma et al., 2013a). Under these conditions THC-COOH was completely degraded after 5 min. Eight TPs were detected, four being intermediate compounds and the other four showing higher persistence along the experiment. As an example, Fig. 2 illustrates the profiles for the seven TPs detected in negative ionization mode 33 h after the chlorination experiment. TP 3C showed the highest absolute area followed by TP 1C, TP 2C and TP 4C, which were still all present in the water sample after 33 h. On the contrary, three of these TPs (5C, 6C and 7C) might act as intermediate compounds, as they were observed after 5 min of chlorination but quickly disappeared to probably yield more persistent TPs. Regarding investigation of real-world water samples, persistent TPs seem to be the most relevant compounds as they may be present in EWW and/or SW with a possible negative impact in the environment.

In addition to the seven TPs detected in negative mode, there was another chlorination TP (8C) observed under positive mode. This compound disappeared after 30 min of chlorination.

Fig. 2SI shows the narrow mass window eXtracted Ion Chromatograms (nw-XICs, 0.02 Da mass window width) corresponding to the (de)protonated molecules obtained after 5 min (TPs 5C, 6C, 7C and 8C) and after 33 h (TPs 1C, 2C, 3C and 4C) of chlorination. Note that for TPs 2C and 3C, the main fragments at *m*/*z* 315.1596 and 397.1418 are shown, as they were more sensitive than the deprotonated molecule. Table 3SI summarizes the information obtained for THC-COOH TPs formed after chlorination. Retention times and exact masses, proposed elemental composition for the (de)protonated TPs and their fragment ions, mass errors (mDa and ppm) and DBE are given. All chlorination TPs contained chlorine atoms, except TP 1C, which was the result of oxidation from the original THC-COOH molecule, consistent with the oxidant properties of NaClO. The number of chlorine atoms (between 1 and 4) was assigned based on the isotopic pattern observed in the mass spectrum.

Fig. 2SIa shows intermediate compounds TP 6C and TP 7C containing four chlorine atoms, some of which could be replaced by hydroxyl groups [OH^-] yielding the persistent TP 2C and TP 3C. Chemical structures could not be proposed for these compounds, as there were too many possibilities to locate the positions of chlorine and hydroxyl groups. Additional analytical techniques, such as NMR or H/D exchange, could be applied for obtaining extra structural information.

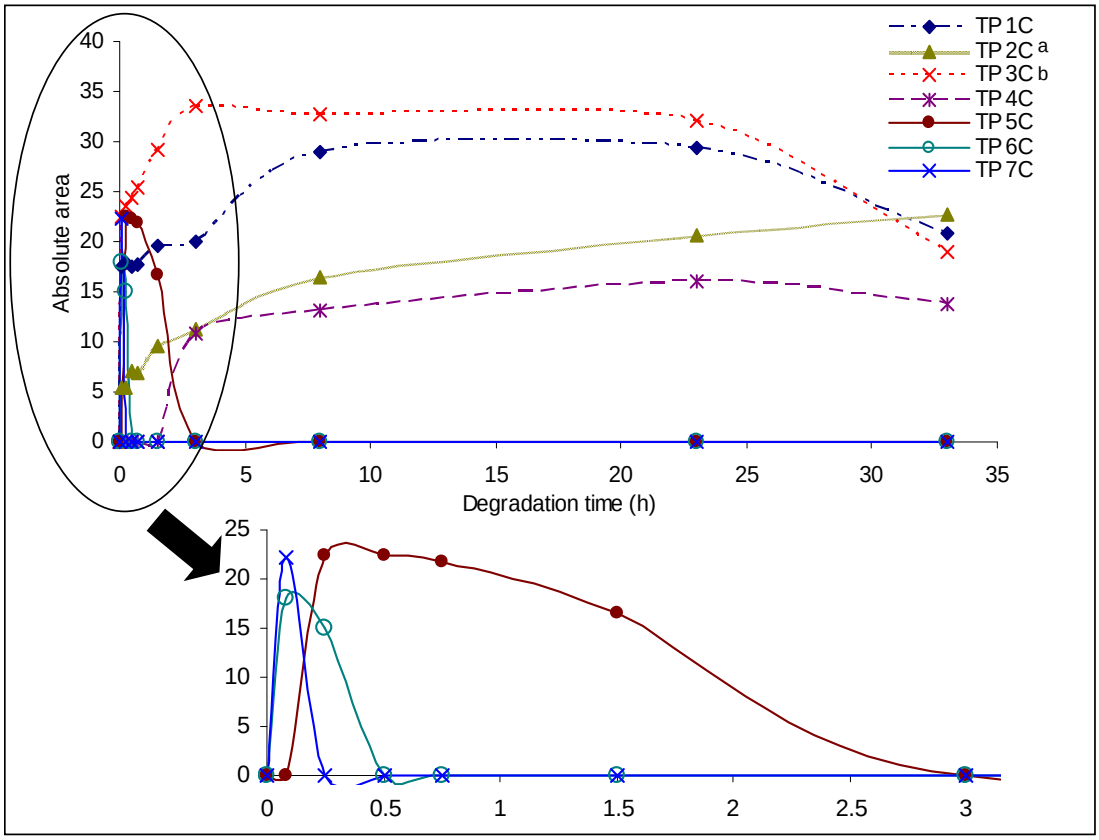


Fig. 2. Degradation curves for TPs obtained after THC-COOH chlorination experiments (QTOF under ESI negative ionization mode).

3.1.3. Photo-degradation (UV)

Trying to simulate another tertiary treatment in WWTP, the ultraviolet photo-degradation of THC-COOH was studied. Fig. 3 shows that THC-COOH was quickly degraded after 30 min of UV exposure, yielding three TPs (TP 1PUV, 2PUV and 3PUV) which also

disappeared completely after 4 h. These results illustrate that UV disinfection process would be an effective removal treatment for THC-COOH in WWTPs. As previously stated, persistent TPs seem to be the most relevant compounds from an environmental point of view. For this reason, in some countries, e.g., Italy, chlorination is being progressively abandoned because of its potential for generating unwanted TPs, and replaced by UV irradiation (Antonelli et al., 2008).

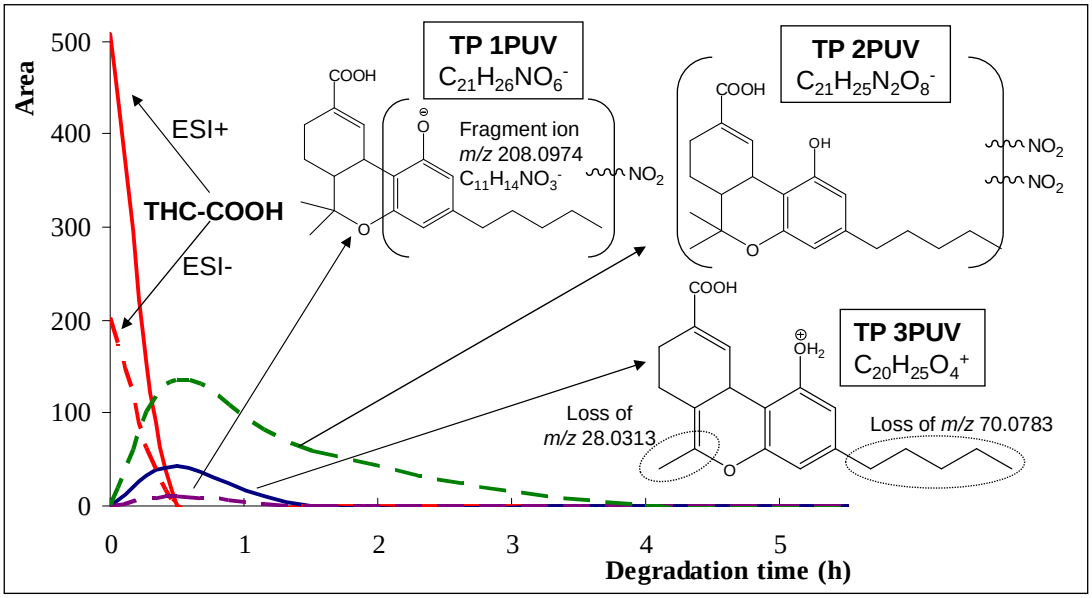


Fig. 3. Degradation curves for TPs 1PUV, 2PUV and 3PUV, detected in positive and negative ion mode, and proposed structures. Ultraviolet photo-degradation experiments.

Among the three TPs found after photo-degradation experiments with UV, one was observed in positive mode while the other two were in negative ionization mode. Elemental compositions, retention times, fragment ions, mass errors and DBEs are summarized in Table 4SI. TP 1PUV (*m/z* 388.1760, $C_{21}H_{26}NO_6^-$, -1.0 mDa) and TP 2PUV (*m/z* 433.1611, $C_{21}H_{25}N_2O_8^-$, +0.2 mDa) eluted at 9.16 and 9.83 min, respectively. Their elemental compositions differed in one and two nitro groups (NO_2) from THC-COOH molecule, respectively. The NO_2 group could easily be introduced since the photo-degradation experiments were performed in surface water from the Mijares River (Castellón province), where relatively high nitrate concentrations are usual due to the wide use of fertilizers in this agricultural area (Hernández et al., 2008, Wick et al.,

2011 and Bijlsma et al., 2013a). Based on fragmentation information, the nitration could have occurred on the benzene ring for the TP 1PUV (see fragment ion at *m/z* 208.0974, $C_{11}H_{14}NO_3$, Fig. 3). For TP 2PUV further information would be necessary to tentatively assign a chemical structure, as the fragment ions available not provide enough information. The photo-degradation product TP 3PUV showed less polarity than the two nitrated TPs, and eluted at 10.13 min. This TP was only detected in positive ionization mode (*m/z* 329.1763). According to the accurate mass data obtained, the elemental composition was assigned to $C_{20}H_{25}NO_4^-$ (ΔmDa = +0.2), which would result from the loss of CH_4 from the THC-COOH molecule. The neutral losses observed in HE mass spectrum (*m/z* 28.0313 C_2H_4 and *m/z* 70.0783 C_5H_{10}, Fig. 3), would imply a demethylation and dehydrogenation in B ring (pos. 5) instead of the aliphatic ring of THC-COOH. The structures proposed for ultraviolet TPs are shown in Fig. 3.

3.1.4. Photo-degradation (sunlight simulation)

Laboratory experiments simulating natural sun light were also performed in order to investigate the possible degradation of THC-COOH in water under environmental conditions. Seven TPs were detected in this case. Fig. 4 shows the degradation rate of the TPs detected in negative ionization mode. As can be seen, THC-COOH was completely degraded after 50 h of irradiation by simulation suntest reactor (equivalent to 8 d of natural sun irradiation). Six TPs were formed, although some of them started to decrease in abundance after 50 h. The most abundant TP, in terms of absolute area, was the TP 1PS which presented its maximum abundance after 45 h of photo-degradation. This TP might suffer a hydrogenation to form the TP 2PS which was still present after 75 h. Similar hydrogenation was observed for TPs 3PS and 4PS. TP 3PS started to increase in abundance after 30 h, just when TP 4PS started to decrease. Finally, TP 5PS and TP 6PS also showed significant maximum intensities, ~300 and ~150 a.u., respectively, after 20 h (Fig. 4).

Another TP was observed only under positive mode. TP 7PS, appeared at 50 h, showing its maximum after 80 h.

It is important to consider these sunlight photo-degradation TPs when investigating the presence of cannabis derivatives in the aquatic environment, as they might be present in environmental waters exposed to solar radiation.

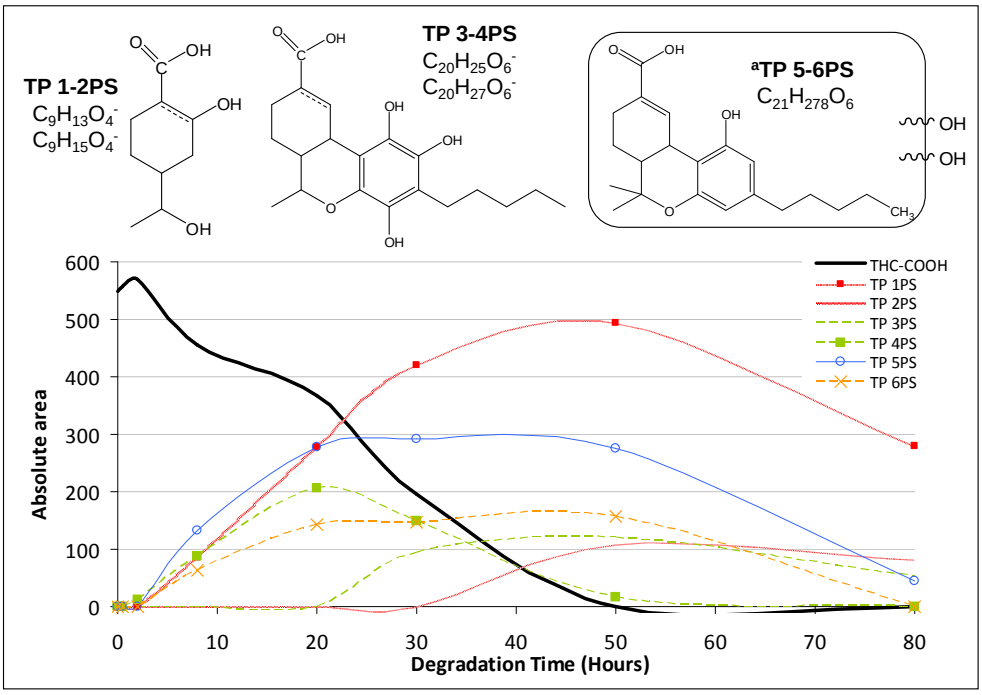


Fig. 4. TPs identified after THC-COOH sunlight photo-degradation experiments (QTOF under ESI negative ionization mode).

Different reactions, such as hydroxylation, demethylation, methylation, or a combination of them, would explain the formation of the seven photo-degraded sunlight TPs. Their elemental compositions, retention times, ionization modes, accurate masses, fragment ions, mass errors and DBEs are shown in Table 5SI. TP 1PS (*m/z* 185.0814, $C_9H_{13}O_4$) could have suffered a hydrogenation to form TP 2PS (*m/z* 187.0970, $C_9H_{15}O_4^-$). The chemical structure of both TPs would correspond to the A ring of THC-COOH, see Fig. 4. The main fragment ions at *m/z* 141.0921 ($C_8H_{13}O_2^-$) and *m/z* 143.1069 ($C_8H_{15}O_2^-$) for TP 1PS and TP 2PS, respectively, indicate a typical loss (CO_2) from a carboxylic acid group. Similar hydrogenation was observed in another two photo-degradation products: TP 4PS (8.06 min, $C_{20}H_{26}O_6$) and TP 3PS (7.45 min, $C_{20}H_{28}O_6$). As Fig. 4 shows, both TPs were detected in negative as well as positive ion modes. Their chemical structures differ from

the THC-COOH molecule in two hydroxylations on the benzene ring plus one demethylation on cyclohexanoate ring. TP 5PS and TP 6PS were photo-degradation TPs sharing the same exact mass but eluting at different retention times (8.64 and 8.80 min, respectively). They were detected in positive (*m*/*z* 377.1964) and negative (*m*/*z* 375.1808) ionization modes too. The chemical structure ($C_{21}H_{28}O_6$) of these isomers might be the result of a double hydroxylation of THC-COOH. However, the positions of the hydroxyl groups were difficult to predict with the information available on fragment ions.

To the authors' best knowledge, these cannabis TPs have not yet been reported in scientific literature.

3.2. Analysis of real-world samples

Five effluent and five influent wastewaters were analyzed by LC-(ESI)-MS/MS (QqQ). Sample treatment consisted of pre-concentration by SPE with Oasis HLB, which has been widely used in the analysis of many different organic pollutants with a wide polarity range. The LC conditions were the same as used in the degradation experiments, in order to obtain comparable retention times. Nineteen TPs and the metabolite THC-COOH were included as target analytes in the method (Table 6SI). For each compound, two SRM transitions were selected based on fragment ions observed by QTOF MS in the degradation experiments. All compounds measured under electrospray positive and negative ionization mode were determined simultaneously in just one injection. In some particular cases, a pseudo-transition was included, i.e. for TPs 1C, 1PS and 2PS, as only one fragment ion (or additional fragment ions of low intensity) was observed in QTOF mass spectra. For the reliable identification of the compounds detected in the samples, the compliance of retention time and *Q*/*q* ratios within maximum tolerances allowed was required (Commission Decision, 2002). As reference standards of TPs were unavailable, the sample vial obtained in the degradation experiments with the highest concentration of analyte was used instead. The use of *Q*/*q* ratios for confirmation was problematic for some TPs, as the value was above 10, which means that the second product ion had very

low abundance, making the use of the second transition less useful. This was reflected in the analysis of some samples as indicated below.

In addition to wastewater samples, several surface waters collected from Spain and The Netherlands were also analyzed.

A summary of the positive findings in IWW, EWW and SW is shown in Table 1. THC-COOH was detected in all IWW (mean concentration 56 ng L^{-1}) and also in EWW, although at a significantly lower levels (mean concentration 8 ng L^{-1}). This is in agreement with the literature, as this major cannabis metabolite is frequently detected in IWW as a result of the wide cannabis consumption (Boleda et al., 2007, Postigo et al., 2010, Bijlsma et al., 2012 and Thomas et al., 2012). The presence of this metabolite in EWW suggests that a partial removal takes place in the WWTPs. No TPs were found in influent wastewater, which might be explained by the fact that still not any treatment has been applied for contaminants removal. However, several TPs were detected in effluent wastewaters. The hydrolysis TP 1H and five sunlight photo-degradation TPs (2PS, 3PS, 4PS, 6PS and 7PS) were detected in effluent samples (between 40% and 80% of positive samples). These TPs were detected using the most abundant transition (Q); however, the second transition was not clearly observed, surely due to low concentration levels of these TPs in the samples. In relation to surface water, the major metabolite THC-COOH was found in 5 out of 10 samples analyzed (the four samples collected from Spain and one sample from The Netherlands). It must be noticed that the Spanish samples were collected in an area (Albufera lake) very close to Valencia. This can explain the presence of this metabolite in all the samples analyzed.

Regarding cannabis TPs, five of these compounds were detected and confirmed in several surface samples: the hydrolysis TP 1H and four sunlight photo-degradation TPs (1PS, 2PS, 5PS and 6PS). The most frequent compound was TP 1PS (m/z 185) which was found in 6 out of 10 surface waters analyzed. However, its identity could not be fully confirmed in 2 samples as Q/q ratio deviation exceeded the maximum tolerance allowed (±30% for ion ratios between 5 and 10) (Commission Decision, 2002). A similar situation was observed

for TP 2PS (*m*/*z* 187), which was detected in 4 samples. It must be noticed that a pseudo MS/MS transition was used for TPs 1PS and 2PS; this would make confirmation using *Q*/*q* ratios more troublesome, due to the lower specificity of these transitions (i.e. the same precursor and product ion selected).

Table 1.Main cannabis metabolite (THC-COOH) and TPs detected in IWW, EWW and SW samples by LC–MS/MS.

	Number of positive findings		
	IWW ($n = 5$)	EWW ($n = 5$)	SW ($n = 10$)
THC-COOH (ESI–/+)	5	5	5
TP 1H (ESI+)	–	3[a]	1
TP 1PS (ESI–)	–	–	6[b]
TP 2PS (ESI–)	–	4	4[b]
TP 3PS (ESI–)	–	3	–
TP 4PS (ESI–)	–	2	8
TP 5PS (ESI+)	–	-	1
TP 6PS (ESI+)	–	2	1
TP 7PS (ESI+)	–	3	–

[a]Underlined, the number of samples where only one transition was observed.
[b]*Q*/*q* ratio deviation >50%.

In order to illustrate the confirmation of positive findings, Fig. 5 shows LC–MS/MS chromatograms for TP 1H detected in a surface water sample from Spain; and for TPs 1PS, 2PS, 5PS and 6PS detected in a surface water sample from The Netherlands. As can be seen, experimental *Q*/*q* ratios were within or close to the maximum deviations and Rt were in agreement with the reference compounds. This allowed us to confirm the identity of the compounds detected, with only the exception of TP 2PS. As previously stated, this might be due to the poor selectivity of the pseudo-SRM transitions used for this compound making the presence of interfering compounds more likely. It is worth noting that non-compliance of *Q*/*q* ratio might lead to false negatives in the case that a matrix-interferent was sharing one of the transitions, thereby producing ion intensity ratios different than expected.

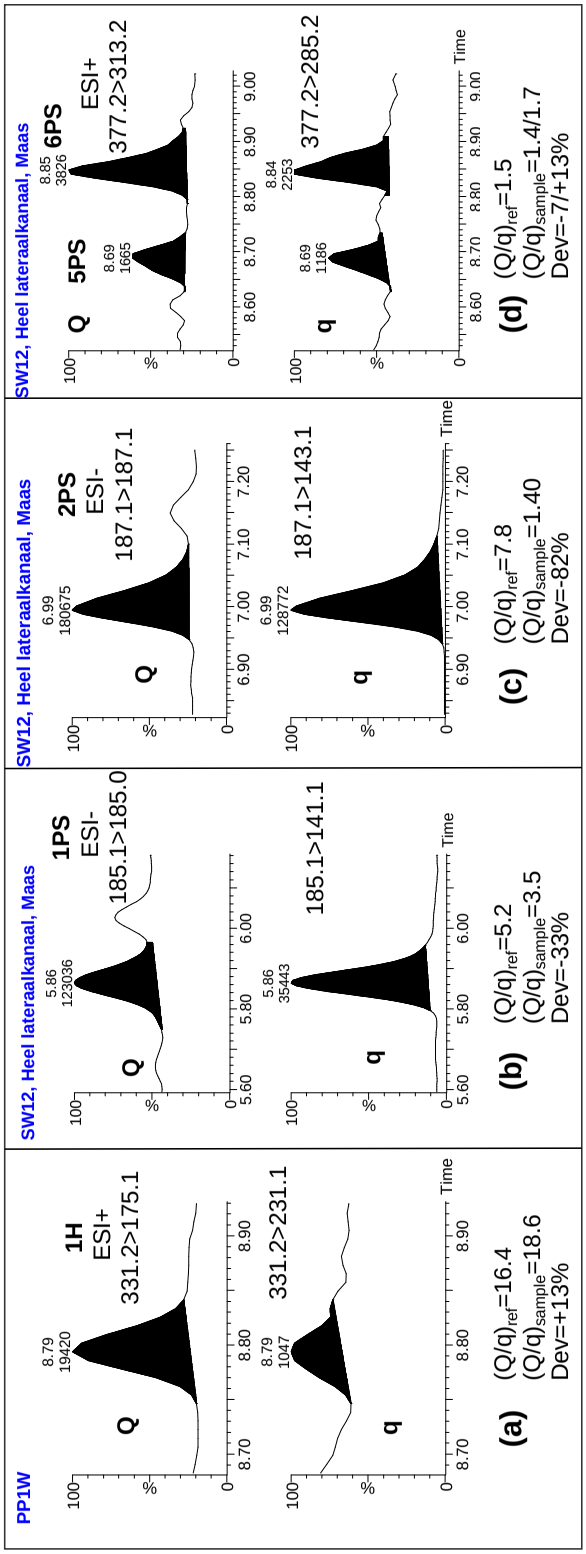


Fig. 5. LC–MS/MS QqQ chromatograms for (a) TP 1H in Spanish surface water; (b) TP 1PS, (c) TP 2PS and (d) TP 5PS and 6PS detected in The Netherlands surface water.

4. CONCLUSIONS

This work reports the behavior of THC-COOH, the major urinary metabolite of cannabis, subjected to hydrolysis, chlorination and photo-degradation (sunlight and UV) experiments under controlled conditions at the laboratory.

Altogether, nineteen THC-COOH TPs (one hydrolysis, eight chlorination, three UV and seven sunlight photo-degradation) were identified by LC-QTOF MS. To the best of our knowledge, most of these TPs have not been reported in the scientific literature yet.

The results from laboratory degradation suggest that UV treatment could be an effective way for removal of THC-COOH in WWTPs, better than chlorination, which generates unwanted TPs.

Analysis of wastewater samples (five IWW and five EWW) and surface waters has allowed us to estimate the removal efficiency of the WWTPs under study as well as to evaluate the presence of cannabis TPs in the aquatic environment. The metabolite THC-COOH was detected in all IWW and EWW samples analyzed, and a removal efficiency of around 86% was estimated. Regarding TPs, none of them were found in influent wastewater, while one hydrolysis and five sunlight photo-degradation TPs were found in effluent wastewaters. In surface waters, THC-COOH was detected in 50% of samples analyzed. The most detected compound (60% of samples) was the sunlight photo-degradation TP 1PS with m/z 185.0814.

The results obtained in the present paper illustrate the importance of investigating TPs in the aquatic environment. Some of the TPs reported in this article, particularly those resulting from sunlight photo-degradation, would need to be investigated in more detail to have a realistic overview of cannabinoids impact in the aquatic environment. Reference standards would be required to unequivocally confirm the identity of these compounds, and to develop analytical methodologies able to accurately quantify their concentration levels in waters.

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REFERENCES

J.L. Acero, F.J. Benítez, F.J. Real, M. González Chlorination of organophosphorus pesticides in natural waters J. Hazard. Mater., 153 (2008), pp. 320–328.

M. Antonelli, V. Mezzanotte, C. Nurizzo Wastewater disinfection by UV irradiation: short and long-term efficiency Environ. Eng. Sci., 25 (2008), pp. 363–373.

J.-. Berset, R. Brenneisen, C. Mathieu Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–MS/MS) Chemosphere, 81 (2010), pp. 859–866.

L. Bijlsma, J.V. Sancho, E. Pitarch, M. Ibáñez, F. Hernández Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater J. Chromatogr. A, 1216 (2009), pp. 3078–3089.

L. Bijlsma, E. Emke, F. Hernández, P. De Voogt Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry Chemosphere, 89 (2012), pp. 1399–1406.

L. Bijlsma, C. Boix, W.M.A. Niessen, M. Ibáñez, J.V. Sancho, F. Hernández Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment Sci. Total Environ., 443 (2013), pp. 200–208.

L. Bijlsma, E. Emke, F. Hernández, P. de Voogt Performance of the linear ion trap Orbitrap mass analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water Anal. Chim. Acta, 768 (2013), pp. 102–110.

C. Boix, M. Ibáñez, J.V. Sancho, W.M.A. Niessen, F. Hernández Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry: degradation experiments J. Mass Spectrom. 48 (2013), pp. 1091-1100.

M.R. Boleda, M.T. Galceran, F. Ventura Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry J. Chromatogr. A, 1175 (2007), pp. 38–48.

M.R. Boleda, M.T. Galceran, F. Ventura Monitoring of opiates, cannabinoids and their metabolites in wastewater, surface water and finished water in Catalonia, Spain Water Res., 43 (2009), pp. 1126–1136.

S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry Anal. Chem., 78 (2006), pp. 8421–8429.

Commission Decision 2002/657/CE of 12 August 2002, Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods of Interpretation of Results.

EPA. Washington, DC, 2004. Primer for Municipal Waste Water Treatment Systems. Document no. EPA 832-R-04-0012.

European Monitoring Centre for Drugs and Drug Addiction. The state of the drugs problem in Europe. EMCDDA Annual Report. 2010 URL: <http://www.emcdda.europa.eu/> (accessed 01.12).

M.I. Farré, S. Pérez, L. Kantiani, D. Barceló Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment TrAC Trends Anal. Chem., 27 (2008), pp. 991–1007.

D. Fatta-Kassinos, M.I. Vasquez, K. Kümmerer Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes – degradation, elucidation of byproducts and assessment of their biological potency Chemosphere, 85 (2011), pp. 693–709.

D. Fatta-Kassinos, S. Meric, A. Nikolaou Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research Anal. Bioanal. Chem., 399 (2011), pp. 251–275.

F. Gosetti, E. Mazzucco, M.C. Gennaro, E. Marengo The challenge of non-target uhplc/ms analysis for the dentification of emerging contaminants in water Environ. Chem. Sust. World (2013) submitted for publication.

F. Hernández, M. Ibáñez, Ó.J. Pozo, J.V. Sancho Investigating the presence of pesticide transformation products in water by using liquid chromatography-mass spectrometry with different mass analyzers J. Mass Spectrom., 43 (2008), pp. 173–184.

F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry Anal. Chim. Acta, 684 (2011), pp. 87–97.

M. Ibáñez, J.V. Sancho, Ó.J. Pozo, F. Hernández Use of quadrupole time-of-flight mass spectrometry in environmental analysis: elucidation of transformation products of triazine herbicides in water after UV exposure Anal. Chem., 76 (2004), pp. 1328–1335.

M. Ibáñez, J.V. Sancho, Ó.J. Pozo, F. Hernández Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazinon as a case study Anal. Bioanal. Chem., 384 (2006), pp. 448–457.

M. Ibáñez, J.V. Sancho, O.J. Pozo, F. Hernández Use of quadrupole time-of-flight mass spectrometry to determine proposed structures of transformation products of the herbicide bromacil after water chlorination Rapid Commun. Mass Spectrom., 25 (2011), pp. 3103–3113.

S. Kern, K. Fenner, H.P. Singer, R.P. Schwarzenbach, J. Hollender Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry Environ. Sci. Technol., 43 (2009), pp. 7039–7046.

F.Y. Lai, C. Ort, C. Gartner, S. Carter, J. Prichard, P. Kirkbride, R. Bruno, W. Hall, G. Eaglesham, J.F. Mueller Refining the estimation of illicit drug consumptions from wastewater analysis: co-analysis of prescription pharmaceuticals and uncertainty assessment Water Res., 45 (2011), pp. 4437–4448.

C. Postigo, M.J. López de Alda, D. Barceló Drugs of abuse and their metabolites in the Ebro River basin: occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation Environ. Int., 36 (2010), pp. 75–84.

J.B. Quintana, R. Rodil, P. López-Mahía, S. Muniategui-Lorenzo, D. Prada-Rodríguez Investigating the chlorination of acidic pharmaceuticals and by-product formation aided by an experimental design methodology Water Res., 44 (2010), pp. 243–255.

G. Skopp, L. Pötsch An investigation of the stability of free and glucuronidated 11-Nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in authentic urine samples J. Anal. Toxicol., 28 (2004), pp. 35–40.

K.V. Thomas, L. Bijlsma, S. Castiglioni, A. Covaci, E. Emke, R. Grabic, F. Hernández, S. Karolak, B. Kasprzyk-Hordern, R.H. Lindberg, M. Lopez de Alda, A. Meierjohann, C. Ort, Y. Pico, J.B. Quintana, M. Reid, J. Rieckermann, S. Terzic, A.L.N. van Nuijs, P. de Voogt Comparing illicit drug use in 19 European cities through sewage analysis Sci. Total Environ., 432 (2012), pp. 432–439.

A.G. Trovó, R.F.P. Nogueira, A. Agüera, C. Sirtori, A.R. Fernández-Alba Photodegradation of sulfamethoxazole in various aqueous media: persistence, toxicity and photoproducts assessment Chemosphere, 77 (2009), pp. 1292–1298.

A.L.N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M.L. de Alda, H. Neels, E. Zuccato, D. Barcelo, A. Covaci Illicit drug consumption estimations derived from wastewater analysis: a critical review Sci. Total Environ., 409 (2011), pp. 3564–3577.

P. Vazquez-Roig, V. Andreu, C. Blasco, Y. Picó SPE and LC–MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (València, Spain) Anal. Bioanal. Chem., 397 (2010), pp. 2851–2864.

W. Weinmann, M. Goerner, S. Vogt, R. Goerke, S. Pollak Fast confirmation of 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) in urine by LC/MS/MS using negative atmospheric-pressure chemical ionisation (APCI) Forensic Sci. Int., 121 (2001), pp. 103–107.

A. Wick, M. Wagner, T.A. Ternes Elucidation of the transformation pathway of the opium alkaloid codeine in biological wastewater treatment Environ. Sci. Technol., 45 (2011), pp. 3374–3385.

SUPPLEMENTARY INFORMATION

Investigation of cannabis biomarkers and transformation products in waters by liquid chromatography coupled to time of flight and triple quadrupole mass spectrometry

THC-COOH is reported in positive (*m/z* 345.2066) and negative (*m/z* 343.1909) ionization modes. The Figure 1SI shows its HE mass spectra and the main fragment ions. In positive ionization mode, the most intense fragment ion at *m/z* 299.2001 corresponds to the loss of formic acid. The other fragments have been justified in our previous works about fragmentation (Bijlsma, et al., 2011). Regarding negative acquisition mode, four low-intense fragment ions were observed. The ion at *m/z* 299 corresponds to the CO_2 loss. The fragment ion at *m/z* 191.1082 ($C_{12}H_{15}O_2^-$, +1.0 mDa) corresponds to the positive 193.1229 ($C_{12}H_{17}O_2^+$, 0.3 mDa). Other fragments ions observed in negative mode at *m/z* 245.1559 ($C_{16}H_{21}O_2^-$, +1.7 mDa) and *m/z* 179.1084 ($C_{11}H_{15}O_2^-$, +1.2 mDa) are illustrated in Figure 1SI.

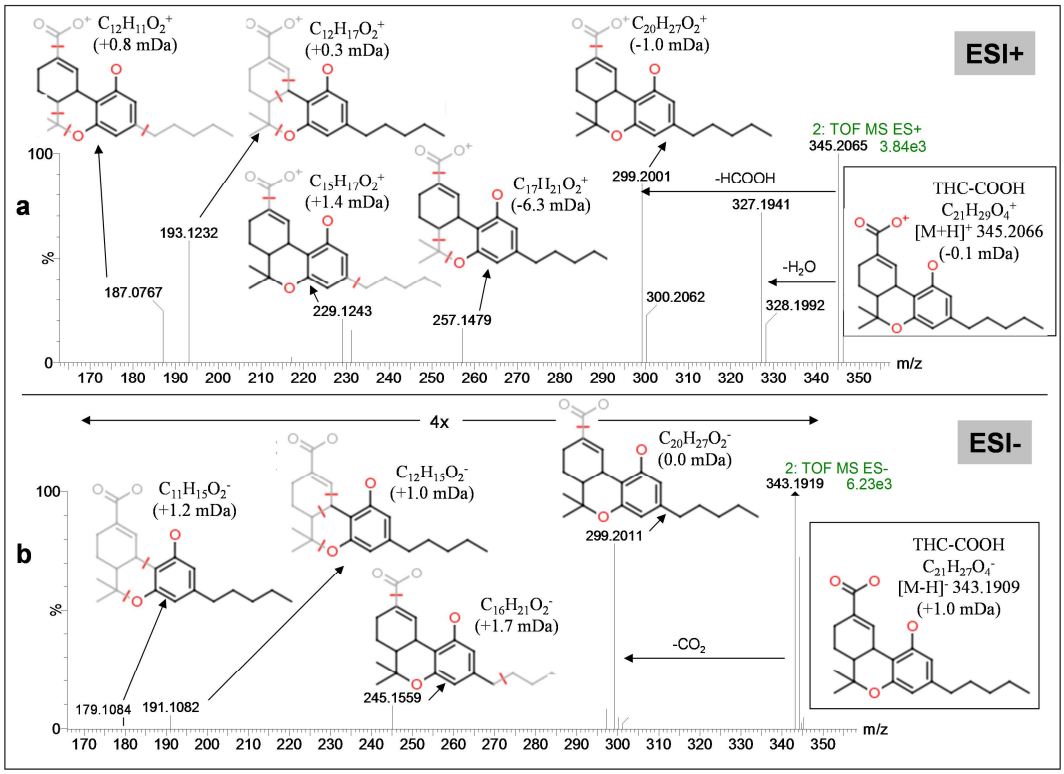


Figure 1SI. QTOF HE spectra of THC-COOH in (a) positive and (b) negative ionization mode; fragment ion structures proposed by MassFragment.

Table 1SI. Characteristics of the surface water used in the degradation experiments.

Characteristics	**Values**
Temperature (ºC, *in situ*)	15.1
pH (*in situ*)	8.5
Conductivity (µS/cm, *in situ*)	810.7
Hardness (expressed as ppm $CaCO_3$)	316.8
Alkalinity (expressed as ppm HCO_3^-)	131.6
Chlorides (expressed as ppm Cl^-)	44.2
Nitrates (expressed as ppm NO_3^-)	3.8
Phosphate (expressed as ppm PO_4^{3-})	2.8
Organic matter (expressed as ppm O_2)	2.2
Sulphates (expressed as ppm SO_4^{2-})	218.7

Table 2SI. Some characteristics of the Castellon WWTP.

Castellon WWTP	
Population served	32,000
Origin	urban and mixed urban and industrial
Average flow rate (m^3/d)	8250
EWW samples	**[a]Treatment**
3 samples	Primary step, biological
1 sample	Primary step, biological, removal Chlorination
1 sample	Primary step, biological, removal UV

[a] Primary step: physical treatment (inc. grit removal). Secondary step: biological treatment (activated sludge). Tertiary step: removal by chlorination or ultraviolet treatment.

Table 3SI. Proposed elemental composition, retention time (min), accurate mass (*m/z*), mass error (mDa, ppm) and double bound equivalent (DBE) of (de)protonated TPs of THC-COOH and their fragments ions obtained in chlorination experiments.

Compound Rt (min)	Ionization mode	Exact mass (*m/z*)	Elemental composition	Mass Error (mDa)	Mass Error (ppm)	DBE
TP 1C	ESI-	167.0708	$C_9H_{11}O_3^-$	0.1	0.6	4.5
6.17		123.0810	$C_8H_{11}O^-$	0.3	2.4	3.5
		95.0861	$C_7H_{11}^-$	1.5	15.8	2.5
TP 2C	ESI-	459.1422	$C_{21}H_{28}O_9Cl^-$	-2.0	-4.4	7.5
7.15		395.1342	$C_{19}H_{23}O_9^-$	-2.1	-5.3	8.5
		315.1596	$C_{19}H_{23}O_4^-$	0.5	1.6	8.5
		253.1592	$C_{18}H_{21}O^-$	2.6	10.3	8.5
TP 3C	ESI-	477.1083	$C_{21}H_{27}O_8Cl_2^-$	-0.4	-0.8	7.5
7.42		433.1185	$C_{20}H_{27}O_6Cl_2^-$	0.9	2.1	6.5
		397.1418	$C_{20}H_{26}O_6Cl^-$	1.3	3.3	7.5
		361.1651	$C_{20}H_{25}O_6^-$	1.4	3.9	8.5
		259.1334	$C_{16}H_{19}O_3^-$	1.6	6.2	7.5
		231.1385	$C_{15}H_{19}O_2^-$	-0.5	-2.2	6.5
		179.0708	$C_{10}H_{11}O_3^-$	0.2	1.1	5.5
TP 4C	ESI-	481.1032	$C_{20}H_{27}O_9Cl_2^-$	1.4	2.9	6.5
7.56		427.1160	$C_{20}H_{24}O_8Cl^-$	2.0	4.7	8.5
		317.1753	$C1_9H_{25}O_4^-$	1.8	5.7	7.5
		199.0970	$C_{10}H_{15}O_4^-$	0.2	1.0	3.5
		167.0708	$C_9H_{11}O_3^-$	1.9	11.4	4.5
		139.0395	$C_7H_7O_3^-$	1.5	10.8	4.5
		95.0497	$C_6H_7O^-$	1.9	20.0	3.5
TP 5C	ESI+	481.0508	$C_{21}H_{25}O_4Cl_4^+$	0.6	1.2	7.5
10.29		374.9958	$C_{16}H_{14}O_4Cl_3^+$	-1.3	-3.5	8.5
		332.9488	$C_{13}H_8O_4Cl_3^+$	-0.2	-0.6	8.5
		314.9383	$C_{13}H_6O_3Cl_3^+$	-0.6	-1.9	9.5
		276.9590	$C_{11}H_8O_2Cl_3^+$	0.6	2.2	6.5
	ESI-	479.0350	$C_{21}H_{21}O_4Cl_4^-$	0.4	0.8	8.5
		407.0817	$C_{21}H_{21}O_4Cl_2^-$	1.6	3.9	10.5
		399.0685	$C_{20}H_{22}O_2Cl_3^-$	-2.0	-5.0	8.5
		363.0919	$C_{20}H_{21}O_2Cl_2^-$	0.3	0.8	9.5
TP 6C	ESI+	499.0613	$C_{21}H_{27}O_5Cl_4^+$	-1.0	-2.0	6.5
10.33		456.9932	$C_{21}H_{17}O_3Cl_4^+$	-1.0	-2.2	11.5
		314.9383	$C_{13}H_6O_3Cl_3^+$	3.4	10.8	9.5
		428.9830	$C_{16}H_{17}O_5Cl_4^+$	-4.2	-9.8	6.5
		395.0736	$C_{21}H_{22}OCl_3^+$	3.3	8.4	9.5
		375.0088	$C_{14}H_{19}O_3Cl_4^+$	0.4	1.1	3.5
	ESI-	497.0456	$C_{21}H_{25}O_5Cl_4^-$	1.7	3.4	7.5
		461.0689	$C_{21}H_{24}O_5Cl_3^-$	2.3	5.0	8.5
		427.1079	$C_{21}H_{25}O_5Cl_2^-$	-0.1	-0.2	8.5
		425.0923	$C_{21}H_{23}O_5Cl_2^-$	2.2	5.2	9.5
		381.1024	$C_{20}H_{22}O_3Cl_2^-$	1.5	3.9	8.5

Table 3SI. (Cont.) Proposed elemental composition, retention time (min), accurate mass (*m/z*), mass error (mDa, ppm) and double bound equivalent (DBE) of (de)protonated TPs of THC-COOH and their fragments ions obtained in chlorination experiments.

Compound Rt (min)	Ionization mode	Exact mass (*m/z*)	Elemental composition	Mass Error (mDa)	Mass Error (ppm)	DBE
TP 7C	ESI+	513.0769	$C_{22}H_{29}O_5Cl_4^+$	-4.2	-8.2	6.5
10.57		445.0740	$C_{21}H_{24}O_4Cl_3^+$	4.7	10.6	8.5
		395.0817	$C_{20}H_{21}O_4Cl_2^+$	0.3	0.8	9.5
		374.9958	$C_{16}H_{14}O_4Cl_3^+$	-1.5	-4.0	8.5
		356.9852	$C_{16}H_{12}O_3Cl_3^+$	-1.3	-3.6	9.5
		314.9383	$C_{13}H_6O_3Cl_3^+$	3.9	12.4	9.5
	ESI-	511.0613	$C_{22}H_{27}O_5Cl_4^-$	1.5	2.9	7.5
		395.1181	$C_{21}H_{25}O_3Cl_2^-$	1.8	4.6	8.5
TP 8C	ESI+	447.0897	$C_{21}H_{26}O_4Cl_3^+$	0.1	0.2	7.5
10.71		393.1024	$C_{21}H_{23}O_3Cl_2^+$	-0.5	-1.3	9.5
		351.0685	$C_{16}H_{22}O_2Cl_3^+$	0.4	1.1	4.5
		325.0398	$C_{16}H_{15}O_3Cl_2^+$	-1.2	-3.7	8.5
		165.0916	$C_{10}H_{13}O_2^+$	0.3	1.8	4.5
		123.0446	$C_7H_7O_2^+$	0.4	3.3	4.5

Table 4SI. Proposed elemental composition, retention time (min), accurate mass (*m/z*), mass error (mDa, ppm) and double bound equivalent (DBE) of (de)protonated TPs of THC-COOH and their fragments ions obtained during UV photo-degradation of experiments.

Compound Rt (min)	Ionization mode	Exact mass (*m/z*)	Elemental Composition	Mass Error (mDa)	Mass Error (ppm)	DBE
TP 1PUV	ESI-	388.1760	$C_{21}H_{26}NO_6^-$	-1.0	-2.6	9.5
9.16		344.1862	$C_{20}H_{26}NO_4^-$	4.0	11.6	8.5
		284.0559	$C_{15}H_{10}NO_5^-$	2.8	9.9	11.5
		236.0923	$C_{12}H_{14}NO_4^-$	0.5	2.1	6.5
		208.0974	$C_{11}H_{14}NO_3^-$	0.9	4.3	5.5
TP 2PUV	ESI-	433.1611	$C_{21}H_{25}N_2O_8^-$	0.2	0.5	10.5
9.83		389.1713	$C_{20}H_{24}N_2O_6^-$	2.4	6.2	9.5
		256.0974	$C_{15}H_{14}NO_3^-$	2.7	10.5	9.5
TP 3PUV	ESI+	329.1753	$C_{20}H_{25}O_4^+$	0.2	0.6	8.5
10.13		301.1440	$C_{18}H_{21}O_4^+$	-1.1	-3.7	8.5
		231.0657	$C_{13}H_{11}O_4^+$	2.5	10.8	8.5
		215.0708	$C_{13}H_{11}O_3^+$	1.2	5.6	8.5
		201.0552	$C_{12}H_9O_3^+$	-1.9	-9.5	8.5

Table 5SI. Proposed elemental composition, retention time (min), accurate mass (*m/z*), mass error (mDa, ppm) and double bound equivalent (DBE) of (de)protonated TPs of THC-COOH and their fragments ions obtained during sunlight photo-degradation experiments.

Compound Rt (min)	Ionization mode	Exact mass (*m/z*)	Elemental composition	Mass Error (mDa)	Mass Error (ppm)	DBE
TP 1PS	ESI-	185.0814	$C_9H_{13}O_4^-$	-0.9	-4.9	3.5
5.83		371.1706	$C_{18}H_{27}O_8^-$	1.1	3.0	5.5
		141.0921	$C_8H_{13}O_2^-$	-0.5	-3.5	2.5
TP 2PS	ESI-	187.0970	$C_9H_{15}O_4^-$	-1.4	-7.5	2.5
6.52		375.2019	$C_{18}H_{31}O_8^-$	1.8	4.8	3.5
		169.0865	$C_9H_{11}O_3^-$	2.2	13.0	3.5
		143.1069	$C_8H_{15}O_2^-$	0.3	2.1	1.5
		125.0966	$C_8H_{11}O^-$	2.4	19.2	2.5
TP 3PS	ESI+	365.1964	$C_{20}H_{29}O_6^+$	0.5	1.4	6.5
7.45		347.1858	$C_{20}H_{27}O_5^+$	0.8	2.3	7.5
		329.1753	$C_{20}H_{25}O_4^+$	1.8	5.5	8.5
		319.1910	$C_{19}H_{27}O_4^+$	2.0	6.3	6.5
		301.1804	$C_{19}H_{25}O_3^+$	0.6	2.0	7.5
		283.1698	$C_{19}H_{23}O_2^+$	1.1	3.9	8.5
		213.1279	$C_{15}H_{17}O^+$	-0.9	-4.2	7.5
		199.1123	$C_{14}H_{15}O^+$	-1.7	-8.5	7.5
	ESI-	363.1808	$C_{20}H_{27}O_6^-$	0.5	1.4	7.5
		319.1909	$C_{19}H_{27}O_4^-$	-0.3	-0.9	6.5
		275.2011	$C_{18}H_{27}O_2^-$	1.7	6.2	5.5
		223.0970	$C_{12}H_{15}O_4^-$	-3.7	-16.6	5.5
		191.1072	$C_{12}H_{15}O_2^-$	0.2	1.0	5.5
TP 4PS	ESI+	363.1808	$C_{20}H_{27}O_6^+$	1.6	4.4	7.5
8.06		265.1076	$C_{14}H_{17}O_5^+$	0.4	1.5	6.5
		247.0970	$C_{14}H_{15}O_4^+$	0.4	1.6	7.5
		229.0865	$C_{14}H_{13}O_3^+$	-0.3	-1.3	8.5
		205.0865	$C_{12}H_{13}O_3^+$	-1.7	-8.3	6.5
		187.0759	$C_{12}H_{11}O_2^+$	-1.2	-6.4	7.5
		179.1072	$C_{11}H_{15}O_2^+$	-1.2	-6.7	4.5
		159.0810	$C_{11}H_{11}O^+$	-1.2	-7.5	6.5
		99.0810	$C_6H_{11}O^+$	0.0	0.0	1.5
	ESI-	361.1651	$C_{20}H_{25}O_6^-$	1.2	3.3	8.5
		343.1545	$C_{20}H_{23}O_5^-$	2.5	7.3	8.5
		299.1647	$C_{19}H_{23}O_3^-$	-0.7	-2.3	8.5
		243.0657	$C_{14}H_{11}O_4^-$	-7.7	-31.7	9.5
		221.0814	$C_{12}H_{13}O_4^-$	0.6	2.7	6.5
		177.0916	$C_{11}H_{13}O_2^-$	1.0	5.6	5.5
		139.0759	$C_8H_{11}O_2^-$	-5.7	-41.0	3.5
		119.0497	$C_8H_8O^-$	-7.7	-64.7	1.5

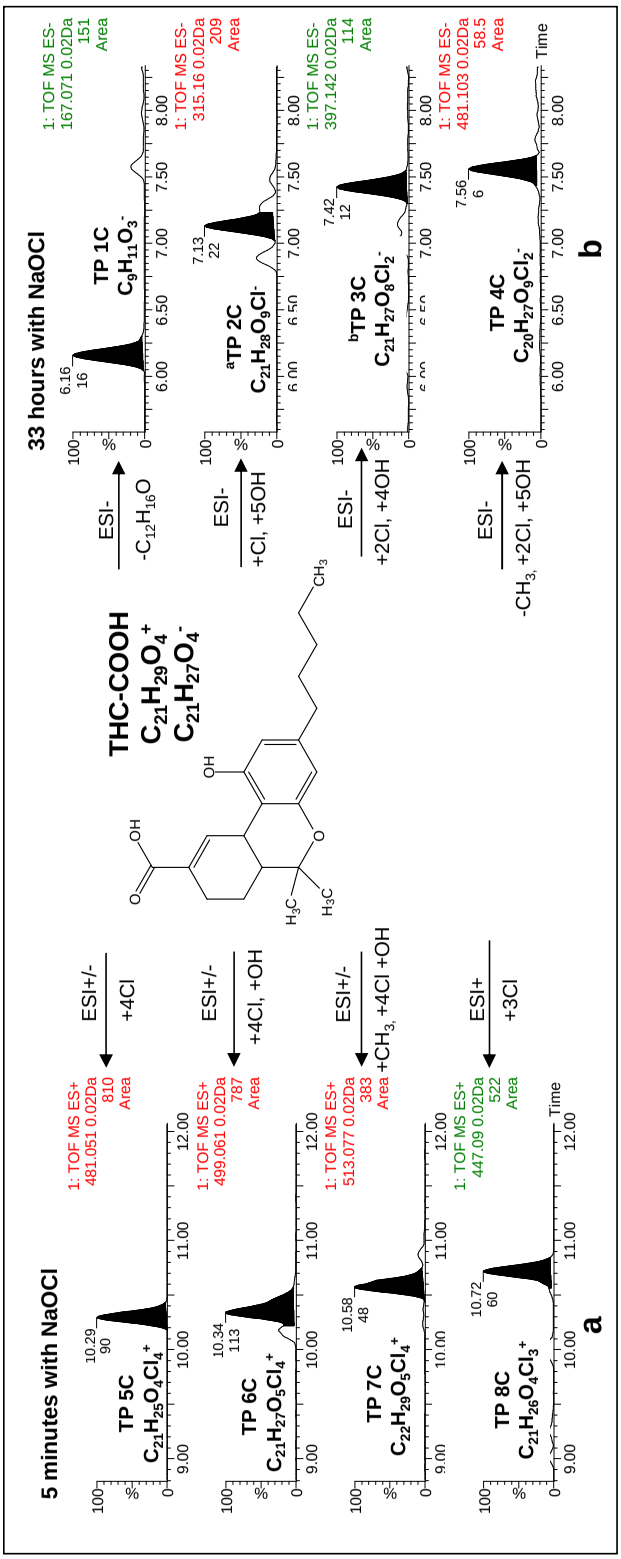
Table 5SI. (Cont.) Proposed elemental composition, retention time (min), accurate mass (*m/z*), mass error (mDa, ppm) and double bound equivalent (DBE) of (de)protonated TPs of THC-COOH and their fragments ions obtained during sunlight photo-degradation experiments.

Compound Rt (min)	Ionization mode	Exact mass (*m/z*)	Elemental composition	Mass Error (mDa)	Mass Error (ppm)	DBE
TP 5-6PS	ESI+	377.1964	$C_{21}H_{29}O_6^+$	-1.4	-3.7	7.5
8.64 - 8.80		359.1858	$C_{21}H_{27}O_5^+$	-0.3	-0.8	8.5
		331.1909	$C_{20}H_{27}O_4^+$	0.2	0.6	7.5
		313.1804	$C_{20}H_{25}O_3^+$	-1.0	-3.2	8.5
		285.1855	$C_{19}H_{25}O_2^+$	-1.7	-6.0	7.5
		243.1385	$C_{16}H_{19}O_2^+$	-1.5	-6.2	7.5
	ESI-	375.1808	$C_{21}H_{27}O_6^-$	2.2	5.9	8.5
		331.1909	$C_{20}H_{27}O_4^-$	-2.5	-7.5	7.5
		287.2011	$C_{19}H_{27}O_2^-$	-4.8	-16.7	6.5
		161.0603	$C_{10}H_9O_2^-$	-7.3	-45.3	6.5
TP 7PS	ESI+	395.2070	$C_{21}H_{31}O_7^+$	0.2	0.5	6.5
8.56		377.1964	$C_{21}H_{29}O_6^+$	3.2	8.5	7.5
		359.1858	$C_{21}H_{27}O_5^+$	-1.6	-4.5	8.5
		331.1910	$C_{20}H_{27}O_4^+$	0.1	0.3	7.5
		313.1804	$C_{20}H_{25}O_3^+$	0.9	2.9	8.5
		285.1855	$C_{19}H_{25}O_2^+$	-0.4	-1.4	7.5
		243.1385	$C_{16}H_{19}O_2^+$	0.2	0.8	8.5
		187.6759	$C_{12}H_{11}O_2^+$	1.1	5.9	7.5

Table 6SI. MS/MS parameters selected for analysis of TTPs by QqQ MS.

Compound	Ionization mode	Retention time (min)	Parent compound	Product ion 1(Q)	[a]CE (eV)	Product ion 2 (q)	[a]CE (eV)	Ion ratio Q/q
THC-COOH	ESI +	9.86	345.0	327.3	15	299.3	20	1.6
	ESI -		343.2	299.2	20	245.2	20	19.8[b]
Hydrolysis								
TP 1H	ESI +	8.80	331.2	175.1	20	231.1	20	16.4
Chlorination								
TP 1C	ESI -	6.17	167.1	*167.1*[c]	5	95.1	20	25.1
TP 2C	ESI -	6.43	395.1	315.2	20	253.2	20	2.6
TP 3C	ESI -	7.80	477.1	361.2	20	231.1	20	8.8
TP 4C	ESI -	7.75	481.1	199.1	20	427.1	20	>100
TP 5C	ESI +	10.40	481.1	277.0	20	315.0	20	1.7
	ESI -		479.0	399.1	20	363.1	20	1.0
TP 6C	ESI +	10.30	499.0	375.0	20	395.1	20	2.8
	ESI -		497.0	461.1	20	381.1	20	0.8
TP 7C	ESI +	10.68	513.1	375.0	20	395.1	20	10.7
	ESI -		511.1	395.1	20	375.0	20	2.7
TP 8C	ESI +	10.71	447.1	393.1	20	123.0	20	1.2
Photo-degradation UV								
TP 1PUV	ESI -	9.16	388.1	236.1	20	284.1	20	-
TP 2PUV	ESI -	9.83	433.2	389.2	20	256.1	20	-
TP 3PUV	ESI +	10.13	329.2	231.1	20	215.1	20	-
Photo-degradation Sunlight								
TP 1PS	ESI -	5.84	185.1	*185.1*[c]	10	141.1	20	5.2
TP 2PS	ESI -	6.68	187.1	*187.1*[c]	10	143.1	20	7.8
TP 3PS	ESI +	7.53	365.2	283.2	20	301.2	20	3.8
	ESI -		363.2	319.2	20	275.2	20	1.5
TP 4PS	ESI +	8.18	363.1	247.1	20	265.1	20	4.3
	ESI -		361.2	177.1	20	221.1	20	2.3
TP 5PS	ESI +	8.68	377.2	313.2	20	285.2	20	1.5
	ESI -		331.2	287.2	20	161.1	20	90.9
TP 6PS	ESI +	8.84	377.2	313.2	20	285.2	20	1.5
	ESI -		331.2	287.2	20	161.1	20	68.4
TP 7PS	ESI +	8.64	395.2	313.2	20	285.1	20	58.0

[a] CE: collision energy. [b] Underlined ion ratios are above 10. These transitions are difficult to observe in the samples due to the low abundance of the product ion 2. In these cases, compliance of Q/q ratio in the samples is rather problematic. [c] Pseudo MS/MS transition.



[a] For TP 2C, main fragment at *m/z* 315.1596 was more sensitive than the deprotonated molecule (*m/z* 459.1422). [b] For TP 3C, main fragment at *m/z* 397.1418 was more sensitive than the deprotonated molecule (*m/z* 477.1083).

Figure 2SI. nw-XICs for the (de)protonated TP molecule and suggested elemental composition obtained. Chlorination experiments after (a) 5 minutes and (b) 33 hours.

REFERENCES

Bijlsma, L., Sancho, J.V., Hernández, F., Niessen, W.M.A., 2011. Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MSE accurate-mass spectra. J. Mass Spectrom. 46, 865-875.

4.3 Discusión de los resultados

En el *Artículo Científico 5* se ha estudiado con detalle el comportamiento del THC-COOH tras los diferentes procesos a los que podría verse sometido en el medio ambiente acuático una vez excretado del cuerpo humano.

Tratando de simular los procesos que se llevan a cabo en una planta de depuración de agua residual, se realizaron experimentos de cloración y de foto-degradación por ultravioleta en condiciones controladas en el laboratorio. Para ello, se tomó una muestra de agua superficial y se fortificó con THC-COOH. Seguidamente se sometieron a las condiciones de degradación utilizando NaOCl para la cloración y una lámpara de mercurio para la foto-degradación. Tras los análisis por UHPLC-QTOF MS se detectaron 8 TPs de cloración y otros 3 de foto-degradación UV.

Por lo que se refiere a los ensayos de cloración, se observó que dicho tratamiento es efectivo para la eliminación del THC-COOH (desapareció completamente a los 5 min.), generando un gran número de TPs desconocidos. Como se muestra en la *Figura 2* del *Artículo Científico 5*, 4 de los 8 TPs de cloración son compuestos persistentes, siendo, por ello, más probable que consigan alcanzar nuestros ríos, lagos o embalses. Además, para la mayoría de los TPs se desconoce su toxicidad y, por consiguiente, también su peligrosidad sobre los organismos existentes en el medio ambiente.

Los ensayos de foto-degradación por ultravioleta mostraron igualmente una efectiva eliminación del metabolito del cannabis *(Figura 3, Artículo Científico 5).* Sin embargo, en este caso se generaban 3 TPs muy inestables, que desaparecían rápidamente. Tras 4 horas de radiación ultravioleta no se detectó la presencia del principal metabolito del cannabis ni tampoco de sus TPs. Con estos resultados se

refuerza la idea reportada por Antonelli et al. (2008), quien apuesta por abandonar los tratamientos de cloración en las plantas de depuración de aguas y sustituirlos por desinfección mediante lámparas ultravioleta.

Dentro de los distintos tipos de degradación que se dan en el medio ambiente, uno de los más importantes, además de la hidrólisis, es el debido al efecto de la radiación solar. Para simular este tipo de foto-degradación se hizo uso de un reactor "*suntest*". Tras los ensayos de degradación y el análisis por LC-QTOF MS, se observó la completa eliminación del THC-COOH después de 50 horas en el reactor. Adicionalmente, se detectaron 7 TPs, que parecieron ser compuestos persistentes y relativamente abundantes. Por ejemplo, el compuesto 1PS, tras 45 horas en el *suntest* (equivalente a 7 días de radiación solar natural), llegó a ser tan abundante como el THC-COOH de partida (t=0 min) (*Figura 4, Artículo Científico 5*). Algunos de estos TPs, como el 4PS, podrían ser compuestos intermediarios de la reacción, ya que se generan en una primera instancia, pero se degradan para dar lugar a otros TPs más persistentes, y por ello, potencialmente más peligrosos.

Se llegaron a detectar hasta 19 TPs del metabolito del cannabis (1 de hidrólisis, 8 de cloración, 3 de foto-degradación UV y 7 de foto-degradación *suntest*), los cuales se intentaron elucidar siguiendo la estrategia detallada en el Capítulo 3. La **Figura 4.3** muestra un esquema a modo de resumen.

La estrategia general para la elucidación de TPs y metabolitos se basó en las siguientes etapas:

1-Análisis de las alícuotas de la muestra sometida a experiencias de degradación mediante QTOF MS, adquiriendo en modo MS^E.

2-Tratamiento de datos con ayuda del software MetaboLynx XS™ (*Waters Corp.*). A partir de la comparación "control" vs "analito" se detectan posibles picos de candidatos a TPs.

3-El espectro de baja energía (LE) proporciona información de la masa exacta de los TPs (generalmente $[M+H]^+$/ $[M-H]^-$).

4-Elucidación tentativa de los TPs detectados. Se estudian los espectros obtenidos tras la adquisición en MS^E (y/o MS/MS en el caso de co-elución). Generalmente, los espectros adquiridos en la función de alta energía de colisión (HE) proporcionan información más útil para la elucidación de las estructuras.

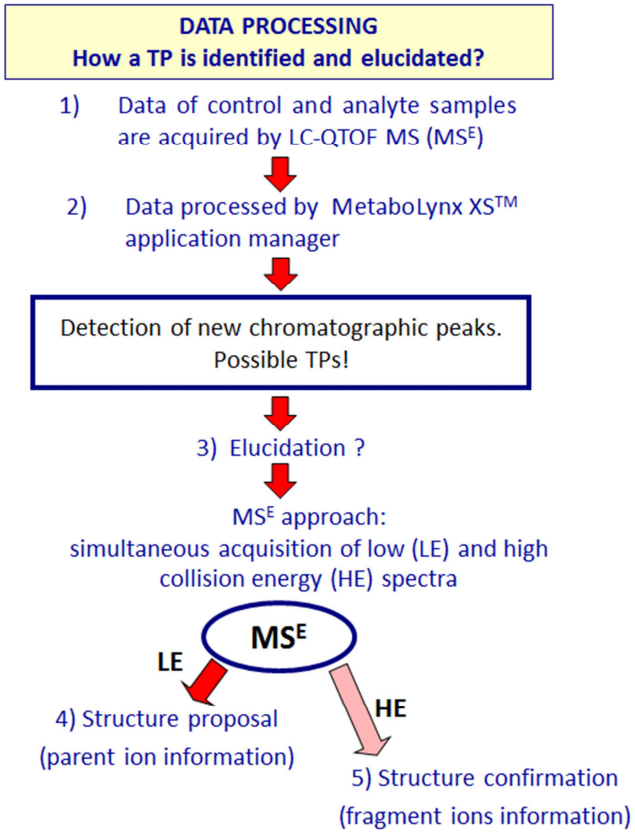


Figura 4.3 Estrategia general para la elucidación de TPs y metabolitos del cannabis.

Para los TPs de foto-degradación 1PS y 2PS esta metodología de elucidación fue complementada con la llamada "barrido de iones precursores" (*Precursor Ion Scan*). Dichos TPs eran moléculas relativamente pequeñas que presentaban poca fragmentación, es decir, generaban poca información estructural. Con el fin de asegurar que las *m*/*z* 185 y 187 se correspondían con los $[M-H]^-$ de los TPs 1PS y 2PS, respectivamente, y no con fragmentos de compuestos inestables en la fuente, se llevó a cabo el análisis con el QqQ en modo de adquisición barrido de iones precursores.

En este modo de adquisición, el primer cuadrupolo (Q_1) hace un barrido de todos los iones que provienen de la fuente, y seguidamente pasan a la celda de colisión, donde, aplicando una energía concreta, se fragmentan. En el segundo cuadrupolo (Q_2), se selecciona sólo el ion producto de interés. Este modo de adquisición resulta útil para detectar compuestos de la misma familia o metabolitos de un mismo analito, ya que el barrido de iones precursores a un ion producto está directamente ligado a una estructura química común.

En la **Figura 4.4** se observan los espectros para el TP 1PS tras la adquisición en modo barrido de iones precursores. Los iones fragmento seleccionados fueron el *m*/*z* 141 y el mismo *m*/*z* 185. Los resultados mostraron que el ion $[M-H]^-$ para el TP 1PS era *m*/*z* 185. Como precursores del *m*/*z* 141 aparecieron *m*/*z* 185 y *m*/*z* 371, pero éste último se corresponde con el dímero. Por otro lado, como precursores del *m*/*z* 185 solo se observó el dímero.

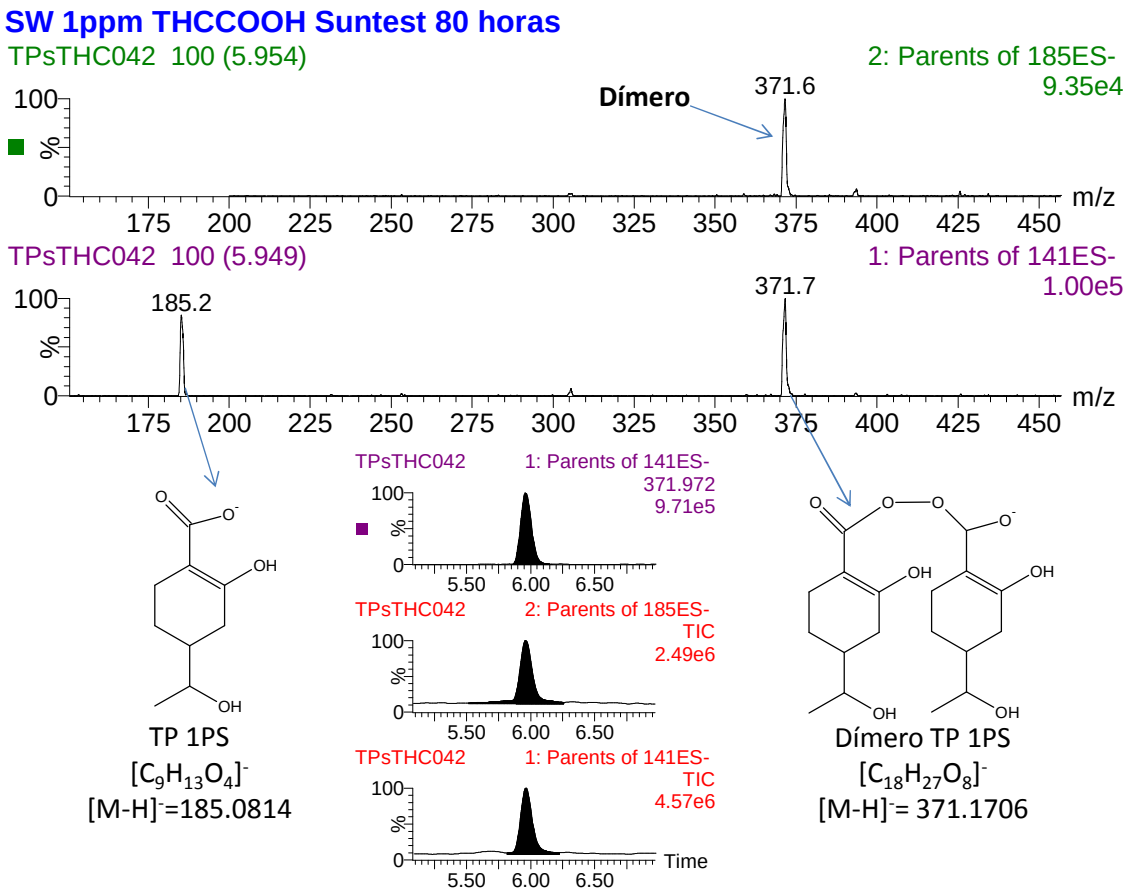


Figura 4.4 Espectros de masa obtenidos tras la adquisición en modo barrido de iones precursores para el TP 1PS.

Finalmente, con el objetivo de investigar la posible presencia de 19 TPs del THC-COOH en el medio ambiente, se analizaron 5 extractos de aguas de influente urbano, 5 de efluente y 10 de superficial. Todas las muestras habían sido sometidas a SPE (Bijlsma, 2014) y analizadas mediante UHPLC-QqQ (MS/MS). El método de análisis que se aplicó para investigar los TPs del cannabis, utilizó las mismas condiciones cromatográficas que en los experimentos de degradación e incluyó las transiciones correspondientes a los iones fragmento observados en los espectros de masas del QTOF, generalmente en la función HE.

Se esperaba, en principio, que estuviesen presentes en las aguas de efluente los TPs procedentes de experimentos de hidrólisis, ultravioleta y cloración; en cambio, en las aguas superficiales cabría esperar, en mayor medida, TPs de foto-degradación por simulación de luz solar, o TPs de hidrólisis.

Realizados los análisis, se observó que el THC-COOH se encontraba en todas las muestras analizadas, aunque en aguas superficiales su concentración era significativamente menor que en los efluentes urbanos. Sin embargo, no se detectó ningún TP en influente urbano, lo cual podría ser lógico, pues dichas aguas brutas aún no han sido sometidas a tratamientos de depuración.

Por lo que se refiere a las aguas de efluente, se observó la presencia del TP de hidrólisis (TP 1H), además de 5 TPs de foto-degradación (luz solar). El TP 2PS fue el compuesto más frecuentemente detectado, encontrándose en 4 de las 5 muestras analizadas.

Por último, en aguas superficiales se pudo detectar la presencia de 6 TPs (1H, 1PS, 2PS, 4PS, 5PS y 6PS). De estos, los TPs de foto-degradación por luz solar 4PS y 1PS estuvieron presentes en 8 y 6 muestras de las 10 analizadas, respectivamente.

De acuerdo con nuestra información, y después de realizar una detallada búsqueda bibliográfica, ninguno de los TPs investigados en este trabajo se había reportado hasta ahora. No obstante, es necesario llevar a cabo estudios más detallados de los TPs generados, obteniendo los patrones de referencia para confirmar inequívocamente su identidad, y sería conveniente investigar en un futuro próximo su peligrosidad para el medio ambiente acuático dado que parecen estar presentes en un notable número de muestras de agua. Todo ello demuestra la importancia de este tipo de investigaciones analíticas en el campo medioambiental.

5
CAPÍTULO

INVESTIGACIÓN DE FÁRMACOS SELECCIONADOS EN AGUAS: EXPERIENCIAS DE BIODEGRADACIÓN

5.1 Introducción

5.2 Artículo Científico 6: *Biodegradation of pharmaceuticals in surface water and during waste water treatment: identification and occurrence of transformation products.* Enviado para su publicación

5.3 Discusión de los resultados

5.1 Introducción

Entre los fármacos más consumidos en España, se encuentran venlafaxina, ibuprofeno, irbesartán, ofloxacino y gemfibrozil, según datos del Sistema Nacional de Salud (IT 4/2011) así como diferentes estudios científicos que reportan elevadas concentraciones de los mismos en aguas residuales españolas (Gracia-Lor, 2011; Ortiz de García, 2013; López-Serna, 2013).

Estudiar el comportamiento de estos fármacos desde que son excretados por el cuerpo humano hasta que alcanzan el medio ambiente es un tema de especial interés, con el fin de conocer, entre otros aspectos, si llegan al medio ambiente como fármaco intacto, como metabolitos o como productos de transformación (Shah, 2010; Dorival-García, 2013; Ferrando-Climent, 2012; Almeida, 2013; Grenni, 2013).

Como se ha explicado en el Capítulo 1, los procesos que normalmente se aplican para el tratamiento de agua residual urbana suelen dividirse en tres categorías: tratamiento primario, secundario y terciario. En el presente Capítulo se investiga el comportamiento de estos fármacos frente al segundo tratamiento, diseñado para

eliminar materia orgánica sobre la base de medidas de BOD (*Biochemical Oxygen Demand*).

La biodegradación que ocurre en una planta de tratamiento se debe a la acción de los microorganismos que provocan la degradación de la materia orgánica, en solución o suspensión, hasta que la BOD del efluente urbano se reduce a niveles aceptables. El proceso de lodos activos, o activados como también se conoce en España, es probablemente el más popular de todos los tratamientos de agua residual. Los microorganismos, en el tanque de aireación, convierten el material orgánico del agua residual en biomasa microbiana y CO_2. El nitrógeno orgánico se convierte en ion amonio o nitrato, mientras que el fósforo orgánico se convierte en fosfato. La degradación de materia orgánica que tiene lugar en una planta de lodos activados puede ocurrir también en aguas superficiales y otros ambientes acuáticos. Sin embargo, debido a la menor concentración de microorganismos, suele requerirse mayor tiempo para degradar el compuesto orgánico.

Considerando el alto consumo de los cinco fármacos previamente mencionados, y que todos ellos presentan una eficiencia de eliminación mayor del 45% tras el paso por las EDARs (Gracia-Lor, 2012; Rosal, 2010; Ortiz de García, 2013; Verlicchi, 2013; Boleda, 2011; Gros, 2010), se podría esperar la presencia de sus productos de transformación en aguas residuales de efluente o incluso en ambientales. Con el objetivo de aportar información sobre este aspecto, surgió el trabajo que se presenta en el *Artículo Científico 6*.

En este trabajo se realizaron experimentos de biodegradación de ibuprofeno, gemfibrozil, irbesartán, ofloxacino y venlafaxina en condiciones controladas en el laboratorio. Se llevaron a cabo dos ensayos de biodegradación: en aguas superficiales y utilizando lodos activados.

El *Artículo Científico 6* se realizó en colaboración con el ***Institute for Biodiversity and Ecosystems Dynamics* (IBED)**, tras una estancia de la doctoranda durante los meses de Noviembre y Diciembre de 2011 en la Universidad de Ámsterdam (Países Bajos). La estancia estuvo supervisada por el Doctor John Parsons, quien dirigió los experimentos de biodegradación utilizando lodos activados. Tras los ensayos llevados a cabo en IBED, las muestras se analizaron mediante UHPLC combinado con QTOF en modo MS^E en el Instituto Universitario de Plaguicidas y Aguas (IUPA) de la Universidad Jaume I, donde también se realizaron experimentos de biodegradación en aguas superficiales.

Tras el procesamiento de los datos utilizando el software MetaboLynx XS™, se detectaron diferentes productos de transformación de los cinco fármacos estudiados. Se intentó elucidar la estructura de estos compuestos con la información obtenida en los espectros de masas a baja y alta energía (o MS/MS caso de los TPs más abundantes), siguiendo la estrategia explicada en los Capítulos 3 y 4.

Por otro lado, también se aplicó la estrategia basada en la búsqueda de *iones fragmento comunes* para detectar la presencia de otros metabolitos/TPs en muestras "reales" (Hernández, 2008; Hernández, 2009). Aprovechando la información de los iones fragmento, tanto de los fármacos como de los TPs observados en las experiencias de biodegradación, se realizó una búsqueda adicional de TPs/metabolitos que compartieran iones fragmento con los mismos. Esta búsqueda se llevó a cabo en muestras de agua residual y superficial previamente sometidas a SPE y analizadas mediante UHPLC-QTOF MS adquiriendo en modo MS^E.

Finalmente, una vez conocida toda esta información, se investigó la presencia de los cinco fármacos y sus TPs mediante un análisis retrospectivo en muestras de agua superficial y residual de efluente analizadas por UHPLC-QTOF MS.

5.2 Artículo Científico 6

BIODEGRADATION OF PHARMACEUTICALS IN SURFACE WATER AND DURING WASTE WATER TREATMENT: IDENTIFICATION AND OCCURRENCE OF TRANSFORMATION PRODUCTS

(Submitted in a Journal)

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ABSTRACT

Venlafaxine, gemfibrozil, ibuprofen, irbesartan and ofloxacin are highly-consumed pharmaceuticals that show considerable removal efficiencies (between 40-98%) in wastewater treatment plants (WWTPs). Consequently, they are expected to generate transformation products (TPs) during wastewater treatment and in surface water (SW) receiving WWTP effluent. In this work, degradation experiments for these five pharmaceuticals have been carried out with SW and WWTP activated sludge under laboratory-controlled conditions to identify their transformation products by liquid chromatography coupled to time-of-flight mass spectrometry (LC-QTOF MS). Initially, 22 pharmaceutical TPs were tentatively identified. A retrospective analysis was performed in effluent wastewater (EWW) and SW samples. Parent compounds as well as several TPs were found in some of the selected EWW and SW samples. Additionally, valsartan and 3 TPs were also detected by searching for common fragments in these water samples. It is important to highlight that some TPs, such as O-desmethyl-venlafaxine and an oxidized gemfibrozil TP, were more frequently found than their corresponding parent compounds. On the basis of these results, it would be recommendable to include these TPs in monitoring programs in order to gain a more realistic understanding of the impact of pharmaceuticals on water quality.

Keywords

Pharmaceuticals, activated sludge biodegradation, effluent wastewater, surface water, transformation/degradation products, time-of-flight mass spectrometry.

1. INTRODUCTION

Large amounts of pharmaceuticals are used around the world and can reach the aquatic environment through urinary excretion and improper disposal (Fatta-Kassinos, Meric and Nikolaou (2011)). Venlafaxine (antidepressant), gemfibrozil (lipid regulator), ibuprofen (anti-inflammatory), irbesartan (angiotensin II receptor antagonist) and ofloxacin (quinolone antibiotic) (Figure 1a) are among the most highly consumed drugs (http://www.msssi.gob.es/en/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf). As a consequence, these compounds have been found in wastewaters (Gilart et al. (2012); Gracia-Lor, Sancho and Hernández (2011); Gracia-Lor et al. (2012a)) and in surface waters (Gracia-Lor, Sancho and Hernández (2011); Gracia-Lor et al. (2012a); Valcárcel et al. (2011); Zuccato, Castiglioni and Fanelli (2005)) since incomplete elimination occurs in wastewater treatment plants. These compounds show different removal rates during wastewater treatment. According to the literature, ibuprofen is the pharmaceutical which shows the highest removal efficiency (~92%) followed by gemfibrozil (~76%), venlafaxine (~50%), ofloxacin (~48%) and irbesartan (~42%) (Ortiz de García et al. (2013); Gracia-Lor et al. (2012b); Verlicchi et al. (2013); Boleda, Galceran and Ventura (2011); Gros et al. (2010)). This elimination can be attributed mainly to biodegradation in combination with sorption processes (Ferrando-Climent et al. (2012)). Therefore, it is expected that potential persistent TPs are generated by transformation/degradation processes in WWTPs, when TPs are sufficient stable or their biodegradation rate is slower than that of a parent compound. The ecotoxicological effects of these TPs are mostly unknown, although some of them could be as, or even more, hazardous than the parent compound, potentially producing negative effects on humans and wildlife (Fatta-Kassinos, Meric and Nikolaou (2011); Kern et al. (2009), Celiz et al. (2009), Escher and Fenner, (2011)). For these reasons, and considering the high consumption of pharmaceuticals, it is important to investigate the possible presence of their TPs in the aquatic environment.

Few articles have reported the degradation or biodegradation (by activated sludge) of irbesartan (Shah, Sahu and Singh (2010)), gemfibrozil (Grenni et al. (2013)), venlafaxine

(Kern et al. (2010); Gasser et al. (2012)) and ofloxacin (Dorival-García et al. (2013)). However, ibuprofen degradation has been frequently studied (Ferrando-Climent et al. (2012); Quintana, Weiss and Reemtsma (2005); Almeida et al. (2013); Marco-Urrea et al. (2009)). Unfortunately, these studies are mainly focused on the determination of degradation rates and not on the identification of transformation compounds generated in degradation processes (de Jongh et al. (2012)).

The vast majority of recent methods for the determination of pharmaceuticals in the aquatic environment are based on the use of liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) using triple quadrupole (QqQ) (Gracia-Lor, Sancho and Hernández (2011); Zuccato, Castiglioni and Fanelli (2005); Ternes, Bonerz and Schmidt (2001); Van Nuijs et al. (2010); González Alonso et al. (2010); Hernando et al. (2007)) or ion trap (IT) (Valcárcel et al. (2011); Rosal et al. (2010); Sousa et al. (2011)) analysers. In the last few years, the presence of pharmaceuticals in environmental samples has also been investigated by LC coupled to Orbitrap MS (Hogenboom, van Leerdam and de Voogt (2009); de Jongh et al. (2012)) or time-of-flight mass spectrometry (TOF MS) (Diaz et al. (2013); José Gómez et al. (2007)). The latter is a powerful tool for screening pharmaceuticals and their TPs in water due to the accurate mass measurements, high resolving power and high full-spectrum acquisition sensitivity (Hernández et al. (2011b); Ibáñez et al. (2009); Nurmi and Pellinen (2011); Quintana, Rodil and Cela (2012)). Moreover, using a hybrid QTOF MS enables the acquisition under MS^E mode, this is, the sequential application of two acquisition functions with different collision energies in a single run. By applying low energy (LE) in the collision cell, fragmentation is minimized, and the information obtained corresponds normally to non-fragmented ions, related to the parent molecule. However, at high collision energy (HE), fragmentation will take place, resulting in abundant fragment ions. The acquisition in MS^E mode allows applying the so-called "fragmentation-degradation" methodology (Hernández et al. (2009)) to search for analyte-related compounds in waters based on the investigation of common fragment ions.

The goal of this work was to carry out a detailed biodegradation study of five pharmaceuticals in surface water and activated sewage sludge. This was performed under laboratory-controlled conditions and the pharmaceutical TPs were identified by LC-QTOF MS. Subsequently, a retrospective analysis was performed in effluent wastewater and surface water samples (previously analyzed by QTOF for screening of pharmaceuticals and drugs of abuse) with the aim of searching for the TPs identified in the laboratory experiments. A different strategy, based on "common fragmentation pathway", was also applied to the water samples, and allowed the further identification of three more TPs.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Reference standards of gemfibrozil, ofloxacin and ibuprofen were obtained from Sigma-Aldrich (Steinheim, Germany). Irbesartan, rac N-desmethyl venlafaxine, D,L-O-desmethyl venlafaxine, 1-hydroxy ibuprofen, rac α-hydroxy ibuprofen and rac 2-hydroxy ibuprofen were purchased from Toronto Research Chemicals (North York, Canada). Venlafaxine hydrochloride was obtained from LGC Promochem (London, UK). Individual stock solutions of 500 mg/L were prepared in HPLC-grade methanol (MeOH) (50 mg/L for ofloxacin).

HPLC-grade MeOH, acetonitrile (ACN), sodium hydroxide (NaOH, 99%) and formic acid (FA, 98-100%) were acquired from Scharlau (Barcelona, Spain). A Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA) was used to obtain the HPLC-grade water. Leucine enkephalin, used as lock mass (*m/z* 556.2771 and *m/z* 554.2615 in positive and negative ion modes, respectively) was purchased from Sigma-Aldrich.

Solid-phase extraction (SPE) cartridges (Oasis-HLB; 3 mL, 60 mg) were purchased from Waters (Milford, MA, USA).

2.2. Activated sewage sludge

Secondary activated sewage sludge, free of heavy particulates and light fractions, was obtained from the Amsterdam West sewage treatment plant on December 4th, 2012. The sludge sample was continuously aerated and stored at room temperature for one week before use in order to reduce the amount of organic matter, and characterised by its total amount of suspended solids (TSS). This parameter was determined by gravimetric analysis (n=3), as described by ESS method 240.2 (1993). Four mineral solutions (see Table 1SI), prepared in demineralised water, were used for the preparation of the mineral medium for biodegradation experiments according to OECD Guideline 301a (OECD, 1992). The mineral solutions were conserved at 4 ^{o}C and inspected to be precipitate free before use.

2.3. Instrumentation

2.3.1. LC-ESI-QTOF MS

An Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) was interfaced to a QTOF mass spectrometer (QTOF Xevo G2, Waters Micromass, Manchester, UK) using an orthogonal Z-spray electrospray interface, operating in both positive and negative ion modes. The LC separation was performed using an Acquity UPLC BEH C18 analytical column (100 x 2.1 mm, 1.7 µm particle size, Waters) at a flow rate of 0.3 mL/min. (*For further details see Supplementary Information SI*).

The data station operating software was MassLynx version 4.1 (Waters).

2.4. Degradation experiments

Biodegradation experiments were performed to investigate the potential for degradation in surface water and by active sludge. The solutions used for biodegradation experiments were individually spiked at 0.5 mg/L with venlafaxine, irbesartan, ibuprofen and gemfibrozil, and at 0.05 mg/L with ofloxacin. These relatively high concentrations allowed better detection and identification of TPs and facilitated the detection of minor TPs. Non-spiked surface water was subjected to the same conditions and used as control sample, to assure that the transformation products formed came from the degradation of the parent pharmaceutical under study. So, differential ions/chromatographic peaks between the degraded sample and the corresponding control sample would correspond, in principle, to transformation products. Of course, it was checked that the analytes were not present in the surface water.

Surface water experiments were performed in samples collected from Mijares River (Valencian Region, Eastern Spain) in 2012 (pH 8.1±0.2) kept in darkness at room temperature. 2-mL aliquots were sampled at different time intervals (0, 1, 2, 4, 7, 10, 14, 18, 24, 30, 44 and 65 days after application) and immediately stored at –20ºC.

Activated sludge experiments were carried out in medium prepared according to Guideline OECD 301a (OECD, 1992) with pH 7.4±0.2. The medium was inoculated with

sludge to give a TSS of 100 mg/L. Solutions (100 mL) were kept in darkness at room temperature, maintaining oxic conditions under continuous shaking (130 rpm). 2-mL aliquots were sampled at different time intervals (0, 5 min, 1, 3, 18 and 27 hours, 2, 4, 11, 16, 21, 25, 30 and 35 days after spiking) and immediately stored at –20ºC.

In both cases, 50 µL were directly injected into the UHPLC-QTOF MS system after centrifugation.

2.5. Identification of TPs by MetaboLynx application manager

The general strategy followed for identification of TPs using UHPLC-ESI-QTOF MS can be found elsewhere (Boix et al. (2013); Bijlsma et al. (2013)).

MetaboLynx XS (an application manager within MassLynx v 4.1) was used to process QTOF MS data. This software compares eXtracted Ion Chromatograms (XICs) of a positive/degraded sample versus a control sample in order to detect, identify and report differential ions/chromatographic peaks which would correspond, in principle, to transformation products (Boix et al. (2013)).

The TPs detected have been named as follows: The first letter corresponds to the initial of the pharmaceutical (eg, "I" for irbesartan, "V" for venlafaxine… except for ibuprofen "Ib"), followed by the degradation process ("SW" for experiments with surface water or "B" for biodegradation experiments using activated sludge). A number (1, 2, 3,…) is added to arrange TPs. Isomeric compounds have the same number but additional final letter (a, b, c…). So, VB1a, corresponds to the transformation product 1 of venlafaxine obtained after biodegradation experiments with AS. Concretely, it refers to isomer a.

2.6. Searching for unknown TPs by common fragmentation pathway

Assuming that most TPs share their fragmentation pathways with the parent pharmaceuticals but also with other TPs (Hernández et al. (2008)), specific nw-XICs at the expected *m/z* fragments were obtained at low (LE) and high (HE) energy from full-spectrum QTOF MS acquisitions. The presence of chromatographic peaks at different retention times (T_R) than the known pharmaceutical compound would indicate the

presence of potential TPs. Using this approach, new compounds have been detected in aquatic samples, EWW and SW (Hernández et al. (2009)).

The TPs detected following this strategy have been named taken into account the initial of the pharmaceutical followed by a number.

2.7. Retrospective QTOF MS analysis of water samples

38 EWW samples were collected from several WWTPs of the Valencian Region (Eastern Spain) from June 2008 to December 2012. Additionally, 18 SW samples were collected from several points located in the same area in March 2010. All water samples had been previously subjected to solid phase extraction and analyzed by LC-QTOF MS for other research purposes (Gracia-Lor et al. (2012a); Hernández et al. (2011a)). Using this technique it is feasible to perform a retrospective evaluation of data at any subsequent time, due to the availability of accurate-mass full-spectrum data generated. Thus, a retrospective analysis was made investigating the presence of the target TPs using ChromaLynx XS application manager (also within MassLynx v 4.1). This software allows applying a "post-target" processing method based on selected exact masses (target list) that permits a rapid and simple reviewing by cataloguing analytes, as function of mass error and retention time deviation. Confirmation of the identity of the compounds detected was based on the accurate *m/z* of the (de)protonated molecule and at least one fragment ion, together with the agreement in retention time (deviation lower than ±2.5%).

3. RESULTS AND DISCUSSION

3.1. Biodegradation rates

3.1.1. Surface water

Figure 1b illustrates the degradation curves (represented as normalised areas respect the area of each compound at t=0) for all studied compounds after 65 days in surface water Significant degradation was only observed for ofloxacin which underwent a removal of around 40% after 44 days. Ibuprofen showed a slight degradation (around 10%) whereas for the rest of compounds, the degradation was negligible.

3.1.2. Activated sludge

In biodegradation experiments, using active sludge (AS) at 100 mg/L of TSS, pharmaceutical elimination seemed to exhibit, in general, a linear decay along the time, except for ibuprofen which followed an exponential decay curve (Figure 1c). Total ibuprofen removal was achieved after 10 days. For gemfibrozil, a removal around 60% was obtained in 7 days, which still increased up to 70% after 35 days. Regarding, ofloxacin and irbesartan removals around 25-30% were obtained after 35 days. Finally, venlafaxine exhibit the lower degradation rate. In general this is consistent with the data reported in the literature (Grenni et al. (2013); Dorival-García et al. (2013); Ferrando-Climent et al. (2012); Almeida et al. (2013)).

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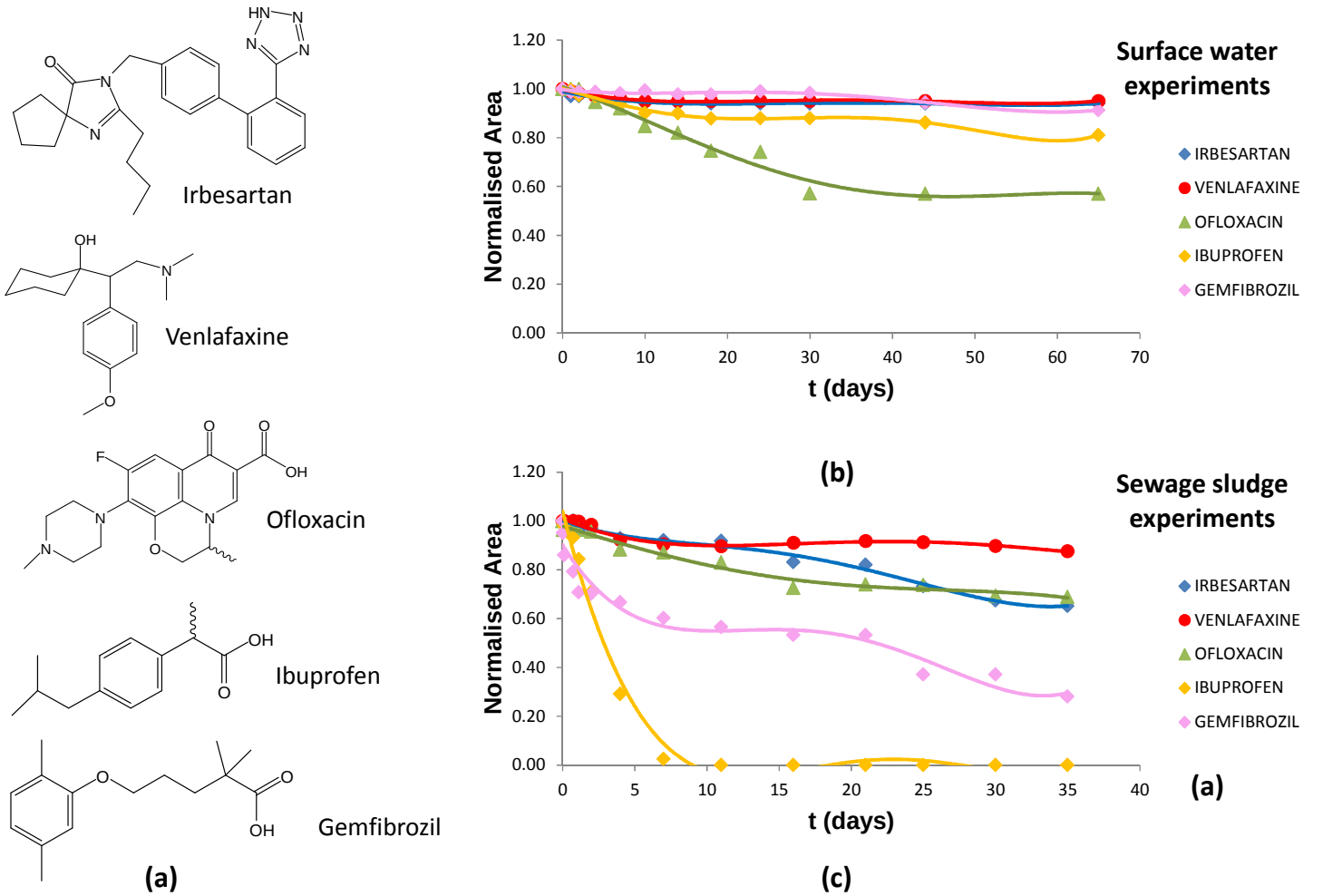

Figure 1. (a) Structures of ofloxacin, gemfibrozil, irbesartan, venlafaxine and ibuprofen. Degradation curves after (b) 44 d in surface water and (c) 14 d with activated sludge.

3.2. Identification of TPs by MetaboLynx application manager

After processing data from the biodegradation experiments using MetaboLynx, several pharmaceutical TPs were found and tentatively identified. Briefly, the identification process was the following. For all compounds detected by MetaboLynx, the accurate mass of protonated/deprotonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Then, possible elemental compositions were calculated using the MassLynx elemental composition calculator with a maximum deviation of 2 mDa from the measured accurate mass. The maximum and minimum parameters were restricted considering the elemental composition of each parent compound. In order to propose a plausible chemical structure for each TP, the fragmentation pathway was studied. For calculating the elemental composition of fragment ions (obtained mainly from HE spectrum), parameters settings were restricted as a function of the calculated elemental composition of the (de)protonated molecule, while for neutral losses no restrictions were applied. In order to avoid spectrum interferences that would complicate the identification process, recognizing which ions are fragments and which are not, becomes mandatory. For this purpose, UHPLC turned valuable for choosing perfectly co-eluting ions. However, and due to the high complexity of the matrix wastewater, MS/MS experiments were also performed to assure that the fragment ions which appeared in full scan experiments arisen from the compound being identified, for which UHPLC played an important role. As it is shown in S.I., no significant differences were found between the MS/MS and the MS^E spectra, demonstrating the usefulness of the MS^E approach even with a very complex matrix such as wastewater *(See SI, Figures 11SI and 21SI).*

Under the conditions used in these experiments, it cannot be excluded that abiotic degradation contributed to the formation of these TPs and further work would be required to elucidate the relative contributions of biotic and abiotic degradation.

Irbesartan. Despite the low removal rate of irbesartan in SW, three minor TPs could be detected (ISW1a, ISW1b and ISW2). In addition, five TPs were identified after biodegradation by AS (IB3a, IB3b, IB4, IB5 and IB6). ISW1a and ISW1b were the major TPs

found in the AS experiments. These TPs are isomeric compounds (*m/z* 447.2508, $[C_{25}H_{31}N_6O_2]^+$) and appeared as a result of irbesartan hydroxylation and hydrogenation in different parts of the molecule *(See SI, Figures 4SI and 5SI)*. ISW2 corresponds to desmethyl-irbesartan (*m/z* 415.2246, $[C_{24}H_{27}N_6O]^+$). Table 2SI summarizes elemental compositions, retention times, fragment ions, mass errors, DBEs and transformation processes for irbesartan and its TPs. Figure 1SIa shows the profile (in a semiLog-linear plot) of the five main irbesartan TPs detected in AS during 35 days. IB3a and IB3b are also isomeric compounds (*m/z* 443.2192, $[C_{25}H_{27}N_6O_2]^+$) showing an oxidation of the irbesartan. IB4 (*m/z* 387.1933 $[C_{22}H_{23}N_6O]^+$) corresponds to a dealkylation of irbesartan. TPs IB5 (*m/z* 459.2145, $[C_{25}H_{27}N_6O_3]^+$) and IB6 (*m/z* 475.2094, $[C_{25}H_{27}N_6O_4]^+$) are formed after oxidation of the parent pharmaceutical and subsequent hydroxylation/s, respectively from irbesartan. ISW1b seems to correspond to one of the three TPs reported by Shah, Sahu and Singh (2010), as both compounds share up to six fragment ions (235.0984, 207.0922, 196.1338, 192.0810, 180.0813 and 168.1388). A plausible chemical structure for each TP is given in S.I. To our knowledge, the other detected irbesartan TPs have not been reported in the literature yet.

Venlafaxine. No TPs were found in SW experiments but six were identified in AS. The formation profiles of the most abundant venlafaxine TPs are shown in Figure 1SIb. VB1a and VB1b were isomeric compounds (*m/z* 264.1964, $[C_{16}H_{26}NO_2]^+$), showing a demethylation of the venlafaxine molecule. It seems that VB1a corresponds to O-desmethyl-venlafaxine, as it still shows a fragment ion at *m/z* 58.0657 ($C_3H_8N^+$). Therefore VB1b might be assigned to N-desmethyl-venlafaxine. Both TPs had been previously reported by Kern et al. (Kern et al. (2010)) in EWW; O-desmethyl TP was also observed in surface water by de Jongh et al. (de Jongh et al. (2012)). The subsequent acquisition of reference standards allowed to confirm the identities of VB1a and VB1b, as retention times and mass spectra were in agreement with O- and N-desmethyl-venlafaxine, respectively. VB3a and VB3b, also isomeric compounds (*m/z* 292.1913, $[C_{17}H_{26}NO_3]^+$), appeared as a result of venlafaxine oxidation in different parts of the molecule. VB2 (*m/z* 294.2069, $[C_{17}H_{28}NO_3]^+$) corresponds to a hydroxylation of venlafaxine. Finally, VB4 (*m/z*

274.1807, $[C_{17}H_{24}NO_2]^+$) is a venlafaxine di-dehydrogenation product. To the authors' best knowledge, these venlafaxine TPs have not yet been reported in scientific literature. Elemental compositions, transformation processes, retention times, fragment ions, mass errors and DBEs are summarized in Table 3SI.

Ofloxacin. Only one TP (OB1) was observed during biodegradation by AS. Its formation during degradation of ofloxacin is illustrated in Figure 1SIc. This TP corresponded to a hydroxylation of ofloxacin (*m/z* 378.1465 $[C_{18}H_{21}N_3O_5F]^+$). Elemental compositions, transformation process, retention times, fragment ions, mass errors and DBEs are summarized in Table 4SI. To our knowledge no reports of ofloxacin TPs have been published in the literature yet.

Ibuprofen. This pharmaceutical was broken down yielding four TPs in SW (IbSW1 and IbSW2a,b,c) and two TPs in AS (IbB3 and IbB4) (Figure 1SId). The TP IbSW1 was the result of a demethylation and dehydrogenation of the ibuprofen structure (*m/z* 189.0916 $[C_{12}H_{13}O_2]^-$). IbSW2a,b,c are isomeric compounds at *m/z* 221.1178 $[C_{13}H_{17}O_3]^-$, eluting at different retention times (6.5, 8.1 and 8.7 min). These compounds resulted from hydroxylation in different parts of the ibuprofen molecule. Two previously reported hydroxylated metabolites (1-hydroxy and 2-hydroxy ibuprofen) (Quintana, Weiss and Reemtsma (2005); Marco-Urrea et al. (2009)) might correspond to two of these TPs IbSW2(a,b,c). After tentative identification of these TPs, three reference standards were acquired (1-hydroxy ibuprofen, rac α-hydroxy ibuprofen and rac 2-hydroxy ibuprofen) for confirmation of their identity. The retention times and mass spectra of rac 2-hydroxy and rac α-hydroxy ibuprofen were in agreement with those of IbSW2a and IbSW2b, respectively. However, in the case of 1-hydroxy ibuprofen, the retention time was not in accordance with that of IbSW2c.

Moreover, AS biodegradation experiments showed IbB3 at *m/z* 165.0552 (-0.7 mDa) with an elemental composition of $[C_9H_9O_3]^-$ obtained after a dealkylation of the butyl group and subsequent hydroxylation. Unfortunately, no fragment ions were observed in the HE spectrum, maybe because it is a relative small and stable molecule, and therefore the

hydroxyl group could not be located. Finally, TP IbB4 was found to have a *m/z* 221.0814 $[C_{12}H_{13}O_4]^-$. This compound shares the nominal mass with TPs IbSW2a,b,c (*m/z* 221), but they have different exact masses as well as retention times. The resolving power and mass accuracy of the QTOF MS allowed differentiation of these compounds. The elemental composition for IbB4 suggests a demethylation, hydroxylation and oxidation of the ibuprofen molecule. Elemental compositions, transformation processes, retention times, fragment ions, mass errors and DBEs are summarized in Table 5SI.

Gemfibrozil. The elimination of this lipid regulator gave a minor TP (GSWB1) in SW. This TP was also observed as a result of biodegradation in AS, although its concentration was almost 15-fold higher in activated sludge (Figure 1SIe). According to its exact mass (*m/z* 279.1232, Table 6SI) the elemental composition of the deprotonated molecule was assigned to $[C_{15}H_{19}O_5]^-$ (+0.9 mDa), which would imply a hydroxylation and oxidation of the gemfibrozil molecule. Figure 2 illustrates the LE and HE spectra for TP GSWB1 with the proposed structures for the fragment ions. On the basis of the fragment ions observed, it might be expected the oxidation to take place in one of the methyl groups of the benzene ring of gemfibrozil. To our knowledge, the TP GSWB1 has not been reported in the literature yet.

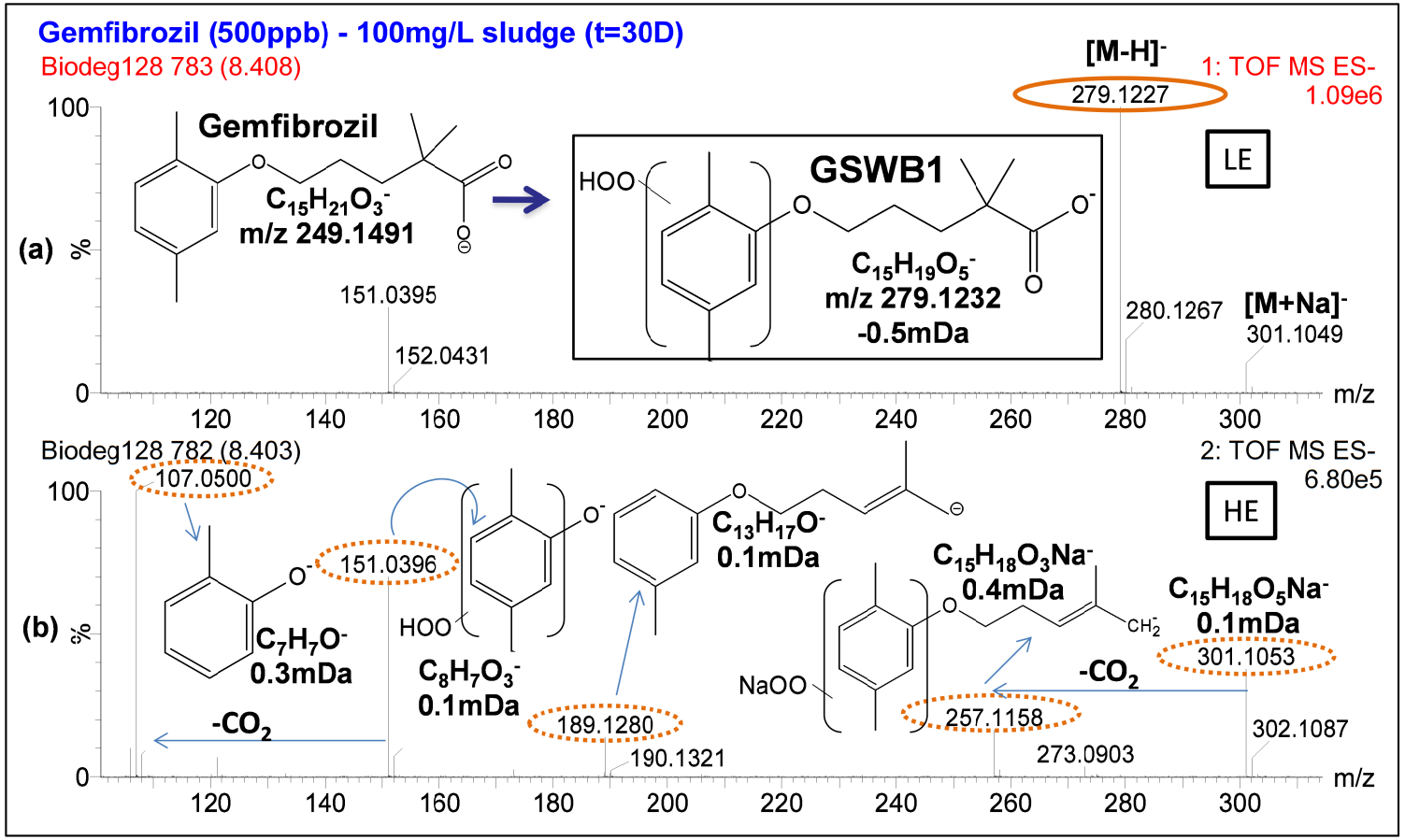

Figure 2. Elucidation of gemfibrozil TP (GSWB1). (a) LE spectrum and (b) HE spectrum with proposed structures for its fragment ions.

3.3. Searching for unknown TPs by common fragmentation pathway

Two unknown compounds (V1 and V2) were detected by common fragmentation pathway with venlafaxine TPs in effluent wastewater. These compounds might be associated with venlafaxine metabolites or to other TPs not found in our biodegradation experiments. V1 shared the exact mass (*m/z* 264.1964) and three fragment ions (*m/z* 58.0657, 93.0704 and 246.1858) with VB1ab TPs, but elute later (at 4.71 min). Retention times of V1 and V2 were re-calculated, since different gradient conditions were used to analyse these samples. For this purpose, T_R of six TPs were measured at each gradient conditions and two equations obtained after their graphical representation, with the correlation coefficients above 0.99 (*For further details see Section 1.3. in SI*).

On the other hand, V2 was detected by common fragmentation with V1. As an example, Figure 3 shows five narrow-mass window extracted ion chromatograms (nw-XICs) for the fragment ions of V1; a new peak appeared (V2) at 4.47 min (the T_R is different than in degradation experiments as they were acquired with different gradients) sharing three of these fragment ions (*m/z* 199.1123, 201.1279 and 157.0653). After investigating the LE function at this retention time, the accurate mass was assigned to *m/z* 258.1869, corresponding to an elemental composition of $[C_{17}H_{24}NO]^+$ (1.1 mDa). Figure 3 shows the LE and HE spectra and the elemental composition assigned to each fragment ion.

As noted above, venlafaxine V1 could have been previously reported by Kern et al. (2010), corresponding to O-desmethyl-venlafaxine and not to N-desmethyl-venlafaxine as it shows a fragment ion at *m/z* 58.0657, $C_3H_8N^+$. Therefore, VB1a and V1 could be explained as a pair of epimer compounds.

Regarding irbesartan, an unknown compound was found by common fragment ions searching in EWW. As can be seen in Figure 2SI, after performing the HE nw-XICs for the fragment ions of irbesartan (at *m/z* 207.0922, 180.0813 and 192.0913) a new peak was observed at 7.94 min. The LE spectrum of the chromatographic peak at this retention time provided the accurate mass of the protonated molecule at *m/z* 436.2350. According to this mass, an elemental composition of $[C_{24}H_{30}N_5O_3]^+$ (0.1 mDa) was assigned. This

chemical formula corresponds to valsartan, a compound also belonging to the angiotensin receptor blockers class. Its identity was confirmed by its retention time, as the reference standard was available at our laboratory (Table 2SI).

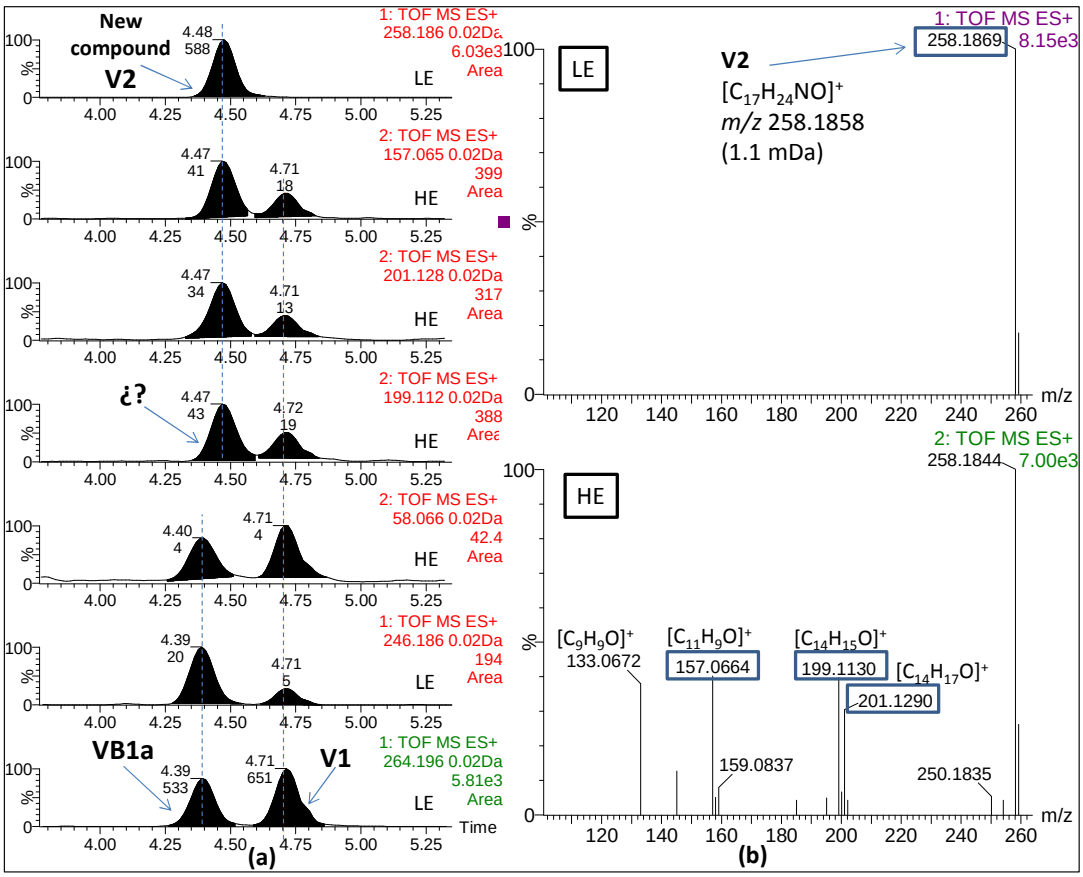

Figure 3. Detection of V1 and V2 venlafaxine metabolites/TPs in EWW by applying the common fragmentation pathway strategy. (a) nw-XICs for $[M+H]^+$ of VB1a, V1 and V2 in LE and four fragment ions in HE. (b) LE and HE spectra of TP V2.

Finally, another ibuprofen-related unknown compound was found in EWW. A new chromatographic peak (Ib1) sharing the same exact mass (*m/z* 221.1178) and two fragment ions (*m/z* 177.1279 and 159.1174) with the ibuprofen TPs IbSW2(abc) was observed at different retention time (6.9 min). According to its accurate mass, an elemental composition of $[C_{13}H_{17}O_3]^-$ (0.2 mDa) was assigned. Its identity was unequivocally confirmed after injecting the 1-hydroxy ibuprofen reference standard, as retention time and mass spectrum were in agreement with Ib1.

Analysis by LC-QTOF under MS^E mode, combined with MetaboLynx application manager and/or common fragmentation pathway strategy, has proven to be a valuable tool for identification of pharmaceutical TPs in waters. The potential of this technique for tentative identification of TPs has been demonstrated, as the subsequent acquisition of reference standards has allowed the unequivocal confirmation of the suggested identity.

3.4. Retrospective search in EWW and SW samples

In order to test whether the TPs identified in this work were present in aquatic samples, a retrospective evaluation of accurate-mass full-acquisition data acquired by QTOF MS was performed. For this purpose, 38 EWW and 18 SW samples, previously analyzed by LC-QTOF MS, were re-processed using ChromaLynx XS software. The database of the compounds investigated contained the elemental composition, fragment ions and retention times of the 6 parent pharmaceuticals, the 22 TPs resulting from the biodegradation experiments and the 3 TPs detected by common fragmentation pathway.

As shown in Table 1, both parent compounds as well as some TPs were found in EWW and SW. Irbesartan was detected in 92% of the EWW and in 39% of SW analyzed. Regarding its TPs, ISW1b, IB3a and IB3b were identified in more than 80% of the EWW samples. The biodegradation product IB5 was also detected in a large number of samples (79%); however, its confirmation was not possible, as no fragment ions that supported its identification were observed. As expected, fewer positive findings were found in SW. As an example, TP IB3a was detected in 84% of the EWW samples and in 22% of the SW samples. The pharmaceutical valsartan was found in 79% and 33% of the EWW and SW samples analyzed, respectively.

Venlafaxine was present in 87% of EWW and 22% of SW samples. It is important to notice that its biodegradation products VB1a and VB1b (O- and N-desmethyl-venlafaxine, respectively) were more frequently detected in effluent wastewater than parent venlafaxine itself. However, VB1b could not be confirmed with fragment ions, presumably due to its low concentration level. Regarding the compounds detected by common

fragment ions, V2 was identified in 87% of the EWW samples and V1 in 58%. The frequencies of detection notably decreased in SW (6-11%).

Table 1. Pharmaceuticals and metabolites/TPs detected in EWW and SW samples after retrospective search in QTOF MS data.

	Positive finding (%)	
	EWW (n=38)	SW (n=18)
Irbesartan	92	39
ISW1b	87	6
IB3a	84	22
IB3b	89	22
IB4	32	11
IB5	79[a]	22[a]
Valsartan	79	33
Venlafaxine	87	22
VB1a	92	17
VB1b	92[a]	17[a]
V1	58	6
V2	87	11
Ofloxacin	82	17
Ibuprofen	11	6
IbSW2a	16	11
IbSW2b	8[a]	0
IbB4	34	50
Ib1	21	6
Gemfibrozil	24	22
GSWB1	71	33

[a] Only the $[M+H]^+$/$[M-H]^-$ was observed.

Ofloxacin was detected in 82% of EWW and 17% of SW samples, but its only TP identified in degradation experiments was not detected in any of the samples.

Regarding ibuprofen, the TP most frequently found in both EWW and SW was IbB4 (34 and 50%, respectively), followed by 1-hydroxy ibuprofen (Ib1, 21% in EWW and 6% in SW) and 2-hydroxy ibuprofen (IbSW2a, 16% in EWW and 11% in SW). Interestingly, all these TPs were more frequently detected than ibuprofen itself.

Similarly, the gemfibrozil biodegradation product GSWB1, was more frequently detected (71% in EWW and 33% in SW) than its parent compound (24% in EWW and 22% in SW). Figure 4 shows a positive finding of gemfibrozil and its TP GSWB1 in EWW. The retention times were re-calculated for samples analyzed under different gradient conditions *(for more details, see section 1.3 in SI)*. In this case, the predicted retention time for this TP under the gradient conditions used in the analysis of the water samples was 11 min, close to 10.82 min observed for this peak. In addition, two fragment ions eluted at the same retention time, supporting the identity of the compound.

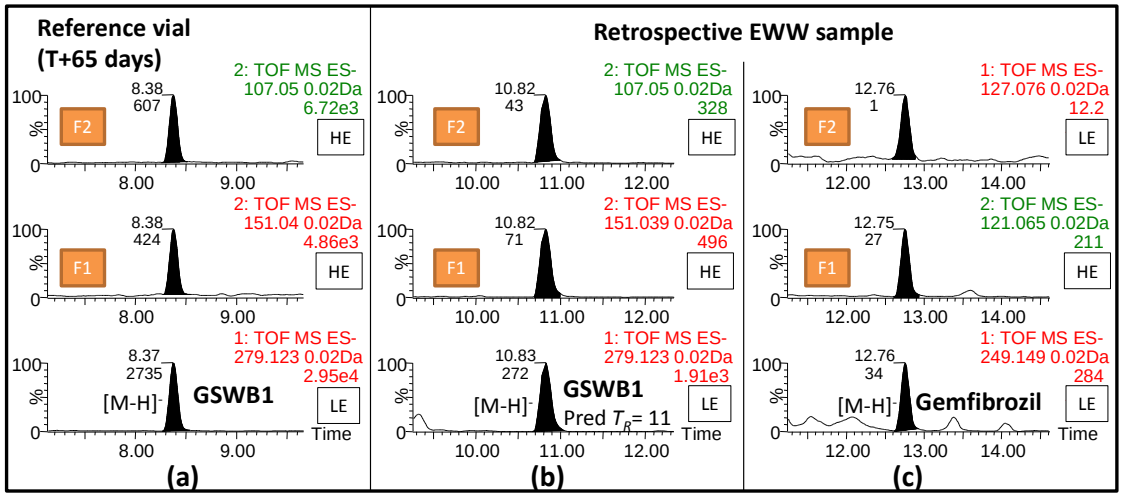

Figure 4. Nw-XICs for $[M-H]^-$ in LE and 2 fragment ions in HE for (a) reference sample vial for gemfibrozil TP GSWB1 obtained in the surface water experiments, (b) positive finding of gemfibrozil TP GSWB1 in EWW. (c) Positive finding of gemfibrozil in the same EWW sample.

Some examples of positive findings are depicted in Figure 5, where selected nw-XICs are shown for irbesartan and 5 of its TPs, valsartan, venlafaxine and 4 TPs, ofloxacin, ibuprofen and 4 TPs, and gemfibrozil and GSWB1 in EWW.

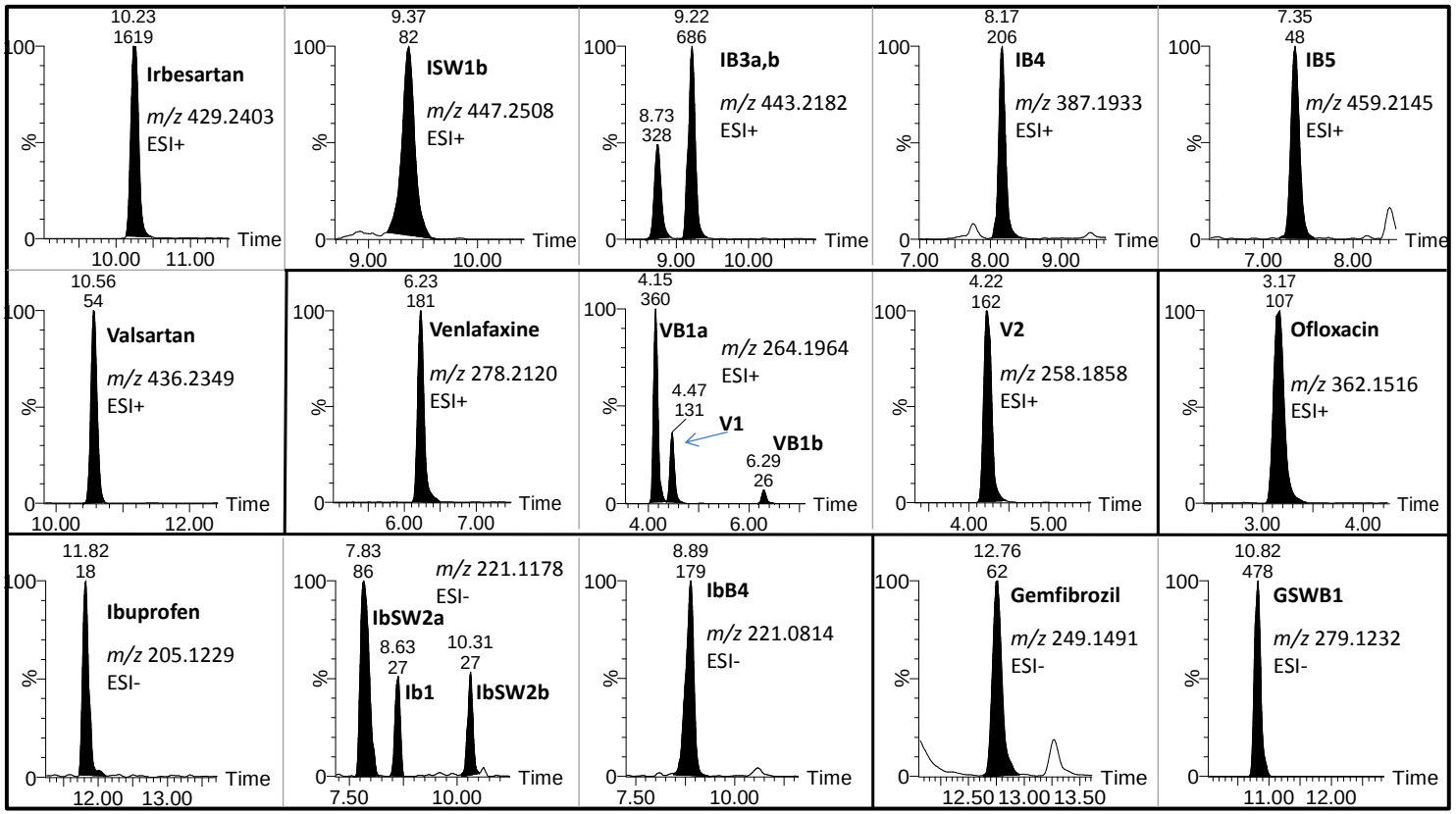

Figure 5. Positive findings of the pharmaceuticals selected and their metabolites/TPs in different EWW samples.

4. CONCLUSIONS

This work reports the degradation of five pharmaceuticals (ibuprofen, ofloxacin, venlafaxine, irbesartan and gemfibrozil) in experiments with surface water and activated sewage sludge under laboratory conditions. A total of 22 TPs were detected and tentatively identified by LC-QTOF MS. Additionally, 2 further venlafaxine TPs and 1 ibuprofen TP were found after applying the strategy based on common fragmentation pathway in effluent wastewater. After tentative identification of the TPs reported in this article, reference standards were acquired (when commercially available) to unequivocally confirm the identity of these compounds. Retrospective evaluation of accurate-mass full-spectrum acquisition data from water samples previously analyzed by QTOF MS showed the presence of parent pharmaceuticals but also 14 transformation products. It is important to highlight that, in some cases, TPs were more frequently detected than the corresponding parent compound. This was the case of ibuprofen degradation products IbSW2, IbB4 and Ib1, and the TP of gemfibrozil, GSWB1. In the light of data reported in this work, it is recommended to include the most relevant TPs, in addition to the parent compounds, in future monitoring programs to gain a more realistic insight of the impact of the presence of pharmaceuticals in the aquatic environment. Data reported in this paper, will facilitate the future development of analytical methodologies for accurate quantification of these TPs in waters (e.g. making use of LC-MS/MS with triple quadrupole).

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REFERENCES

Almeida, B., Oehmen, A., Marques, R., Brito, D., Carvalho, G., Barreto Crespo, M. T. 2013. Modelling the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) by activated sludge and a pure culture. Bioresource Technology 133, 31-37.

Bijlsma, L., Boix, C., Niessen, W. M. A., Ibáñez, M., Sancho, J. V., Hernández, F. 2013. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. Science of the Total Environment 443, 200-208.

Boix, C., Ibáñez, M., Sancho, J.V., Niessen, W.M.A., Hernández, F., 2013. Investigating the presence of omeprazole in waters by liquid chromatography coupled to low and high resolution mass spectrometry: Degradation experiments. Journal of Mass Spectrometry 48 (10), 1091-1100.

Boleda, M. R., Galceran, M. T., Ventura, F. 2011. Behavior of pharmaceuticals and drugs of abuse in a drinking water treatment plant (DWTP) using combined conventional and ultrafiltration and reverse osmosis (UF/RO) treatments. Environmental Pollution 159 (6), 1584-1591.

de Jongh, C.M., Kooij, P.J.F., de Voogt, P., ter Laak, T.L . 2012. Screening and human health risk assessment of pharmaceuticals and their transformation products in Dutch surface waters and drinking water. Science of the Total Environment 427, 70-77.

Celiz, M.D., Tso, J., Aga, D.S., 2009. Pharmaceutical metabolites in the environment: analytical challenges and ecological risks. Environmental Toxicology and Chemistry 28, 2473–84.

Diaz, R., Ibáñez, M., Sancho, J. V., Hernández, F. 2013. Qualitative validation of a liquid chromatography-quadrupole-time of flight mass spectrometry screening method for organic pollutants in waters. Journal of Chromatography A 1276, 47-57.

Dorival-García, N., Zafra-Gómez, A., Navalón, A., González-López, J., Hontoria, E., Vílchez, J. L. 2013. Removal and degradation characteristics of quinolone antibiotics in laboratory-scale activated sludge reactors under aerobic, nitrifying and anoxic conditions. Journal of Environmental Management 120, 75-83.

Escher, B.I., Fenner, K., 2011. Recent advances in environmental risk assessment of transformation products. Environmental Science & Technology 45, 3835–47.

Fatta-Kassinos, D., Meric, S., Nikolaou, A. 2011. Pharmaceutical residues in environmental waters and wastewater: Current state of knowledge and future research. Analytical and Bioanalytical Chemistry 399 (1), 251-275.

Ferrando-Climent, L., Collado, N., Buttiglieri, G., Gros, M., Rodriguez-Roda, I., Rodriguez-Mozaz, S., Barceló, D. 2012. Comprehensive study of ibuprofen and its metabolites in

activated sludge batch experiments and aquatic environment. Science of the Total Environment 438, 404-413.

Gasser, G., Pankratov, I., Elhanany, S., Werner, P., Gun, J., Gelman, F., Lev, O. 2012. Field and laboratory studies of the fate and enantiomeric enrichment of venlafaxine and O-desmethylvenlafaxine under aerobic and anaerobic conditions. Chemosphere 88 (1), 98-105.

Gilart, N., Marcé, R. M., Borrull, F., Fontanals, N. 2012. Determination of pharmaceuticals in wastewaters using solid-phase extraction-liquid chromatography-tandem mass spectrometry. Journal of Separation Science 35 (7), 875-882.

González Alonso, S., Catalá, M., Maroto, R. R., Gil, J. L. R., de Miguel, Á. G., Valcárcel, Y. 2010. Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain). Environment International 36 (2), 195-201.

Gracia-Lor, E., Martínez, M., Sancho, J. V., Peñuela, G., Hernández, F. 2012a. Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high performance liquid-chromatography-tandem mass spectrometry. Talanta 99, 1011-1023.

Gracia-Lor, E., Sancho, J. V., Serrano, R., Hernández, F. 2012b. Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia. Chemosphere 87 (5), 453-462.

Gracia-Lor, E., Sancho, J. V., Hernández, F. 2011. 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 (16), 2264-2275.

Grenni, P., Patrolecco, L., Ademollo, N., Tolomei, A., Barra Caracciolo, A. 2013. Degradation of Gemfibrozil and Naproxen in a river water ecosystem. Microchemical Journal 107, 158-164.

Gros, M., Petrovic, M., Ginebreda, A., Barceló, D. 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environment International 36 (1), 15-26.

Hernández, F., Bijlsma, L., Sancho, J. V., Díaz, R., Ibáñez, M. 2011a. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. Analytica Chimica Acta 684 (1-2), 87-97.

Hernández, F., Ibáñez, M., Gracia-Lor, E., Sancho, J. V. 2011b. Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater. Journal of Separation Science 34 (24), 3517-3526.

Hernández, F., Grimalt, S., Pozo, Ó. J., Sancho, J. V. 2009. Use of ultra-high-pressure liquid chromatography-quadrupole time-of-flight MS to discover the presence of pesticide metabolites in food samples. Journal of Separation Science 32 (13), 2245-2261.

Hernández, F., Sancho, J. V., Ibáñez, M., Grimalt, S. 2008. Investigation of pesticide metabolites in food and water by LC-TOF-MS. Trends in Analytical Chemistry 27 (10), 862-872.

Hernando, M. D., Gómez, M. J., Agüera, A., Fernández-Alba, A. R. 2007. LC-MS analysis of basic pharmaceuticals (beta-blockers and anti-ulcer agents) in wastewater and surface water. Trends in Analytical Chemistry 26 (6), 581-594.

Hogenboom, A.C.; van Leerdam, J.A.; de Voogt, P. 2009. Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-LTQ FT Orbitrap mass spectrometry. Journal of Chromatography A 1216, 510–519.

Ibáñez, M., Guerrero, C., Sancho, J. V., Hernández, F. 2009. Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1216 (12), 2529-2539.

José Gómez, M., Malato, O., Ferrer, I., Agüera, A., Fernández-Alba, A. R. 2007. Solid-phase extraction followed by liquid chromatography-time-of-flight- mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study. Journal of Environmental Monitoring 9 (7), 719-729.

Kern, S., Baumgartner, R., Helbling, D. E., Hollender, J., Singer, H., Loos, M. J., Schwarzenbach, R. P., Fenner, K. 2010. A tiered procedure for assessing the formation of biotransformation products of pharmaceuticals and biocides during activated sludge treatment. Journal of Environmental Monitoring 12 (11), 2100-2111.

Kern, S., Fenner, K., Singer, H. P., Schwarzenbach, R. P., Hollender, J. 2009. Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry. Environmental Science and Technology 43 (18), 7039-7046.

Marco-Urrea, E., Pérez-Trujillo, M., Vicent, T., Caminal, G. 2009. Ability of white-rot fungi to remove selected pharmaceuticals and identification of degradation products of ibuprofen by Trametes versicolor. Chemosphere 74 (6), 765-772.

Nurmi, J. and Pellinen, J. 2011. Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry. Journal of Chromatography A 1218 (38), 6712-6719.

OECD Guidelness for the Testing of Chemicals - Test no. 301 Ready Biodegradability. Organization for Economic Cooperation and Development (OECD). 1992.

Ortiz de García, S., Pinto Pinto, G., García Encina, P., Irusta Mata, R. 2013. Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. Science of the Total Environment 444, 451-465.

Prescription data: IT del Sistema Nacional de Salud Volumen 35, Nº 4/2011 http://www.msssi.gob.es/biblioPublic/publicaciones/recursos_propios/infMedic/docs/SubgruposATCvol35n4.pdf.

Quintana, J. B., Rodil, R., Cela, R. 2012. Reaction of β-blockers and β-agonist pharmaceuticals with aqueous chlorine. Investigation of kinetics and by-products by liquid chromatography quadrupole time-of-flight mass spectrometry. Analytical and Bioanalytical Chemistry 403 (8), 2385-2395.

Quintana, J. B., Weiss, S., Reemtsma, T. 2005. Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. Water Research 39 (12), 2654-2664.

Rosal, R., Rodríguez, A., Perdigón-Melón, J. A., Petre, A., García-Calvo, E., Gómez, M. J., Agüera, A., Fernández-Alba, A. R. 2010. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Research 44 (2), 578-588.

Shah, R. P., Sahu, A., Singh, S. 2010. Identification and characterization of degradation products of irbesartan using LC-MS/TOF, MSn, on-line H/D exchange and LC-NMR. Journal of Pharmaceutical and Biomedical Analysis 51 (5), 1037-1046.

Sousa, M. A., Gonçalves, C., Cunha, E., Hajšlová, J., Alpendurada, M. F. 2011. Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS. Analytical and Bioanalytical Chemistry 399 (2), 807-822.

Ternes, T., Bonerz, M., Schmidt, T. 2001. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography–electrospray tandem mass spectrometry. Journal of Chromatography A 938 (1-2), 175-185.

Valcárcel, Y., González Alonso, S., Rodríguez-Gil, J. L., Gil, A., Catalá, M. 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84 (10), 1336-1348.

Van Nuijs, A. L. N., Tarcomnicu, I., Simons, W., Bervoets, L., Blust, R., Jorens, P. G., Neels, H., Covaci, A. 2010. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater. Analytical and Bioanalytical Chemistry 398 (5), 2211-2222.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., Al Aukidy, M., Zambello, E. 2013. Removal of selected pharmaceuticals from domestic wastewater in an activated sludge system followed by a horizontal subsurface flow bed - Analysis of their respective contributions. Science of the Total Environment 454-455, 411-425.

Wisconsin State Laboratory Hygiene. ESS Method 340.2: Total Suspended Solids, Mass Balance (Dried at 103-105ºC). Volatile Suspended Solids (Ignited at 550 ºC). Wisconsin, USA:s.n., 1993.

Zuccato, E., Castiglioni, S., Fanelli, R. 2005. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. Journal of Hazardous Materials 122 (3), 205-209.

SUPPLEMENTARY INFORMATION

Biodegradation of pharmaceuticals in surface water and during waste water treatment: Identification and occurrence of transformation products

1.1. Reagents and chemicals

Table 1SI. Stock solutions used for the preparation of mineral media.

Mineral stock solution	contents
1	8.5 g/L potassium dihydrogen orthophosphate (KH_2PO_4) 21.75 g/L dipotassium hydrogen orthophosphate (K_2HPO_4) 33.40 g/L disodium hydrogen orthophosphate dehydrate ($Na_2HPO_4 \cdot 2H_2O$) 0.5 g/L ammonium chloride (NH_4Cl)
2	27.50 g/L calcium chloride, anhydrous ($CaCl_2$)
3	22.50 g/L magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)
4	0.25 g/L iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)

1.2. Instrumentation

LC-ESI-QTOF MS

The mobile phases used were (A) H_2O and (B) MeOH, both containing 0.01% (v/v) FA. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10%; 9 min, 90%; 11 min, 90%; 11.1 min, 10%; 14 min, 10%. The column and sample temperatures were kept at 40°C and 5ºC, respectively. The injection volume was 50 µL. Nitrogen (Praxair, Valencia, Spain) was used as both drying gas and nebulising gas. The gas flow rate was set at 1000 L/h. The resolution of the TOF mass spectrometer was ~ 20,000 at full width half maximum (FWHM) at *m/z* 556. MS data were acquired over an *m/z* range of 50–1200, using a scan time of 0.3 s. The MCP detector potential was set to 3450 V. Capillary voltages of 0.7 kV and -1.7 kV were used in positive and negative ionization modes, respectively. A cone voltage of 25 V was applied. The collision gas was argon (99.995%, Praxair). The interface temperature was set to 650 ºC and the source temperature to 130 ºC. For MS^E experiments, two simultaneous acquisition functions were created: the low-energy (LE) function, with a collision energy of 4 eV, and the high energy (HE) function,

with a collision energy ramp ranging from 15 to 40 eV. The same cone voltage (25 V) and collision energy ramp were used for additional MS/MS experiments, when necessary.

Calibration of the mass-axis from *m/z* 50 to 1200 was conducted daily with a 1:1 mixture of 0.05M NaOH:/5% (v/v) HCOOH, diluted (1:25) with water/ACN (20:80 v/v).

For automated accurate mass measurement, leucine enkephalin (2 mg/L) in ACN/water (50/50) at 0.1% HCOOH was used as lockmass and pumped at 20 µL/min through the lock-spray needle. The leucine enkephalin $[M+H]^+$ ion (*m/z* 556.2771) and its fragment ion (*m/z* 278.1141) were used in positive ionization mode for recalibrating the mass axis and to ensure a robust accurate mass measurement over time. In the negative, the selected ions were $[M-H]^-$ ion (*m/z* 554.2615) and its fragment ion (*m/z* 236.1035).

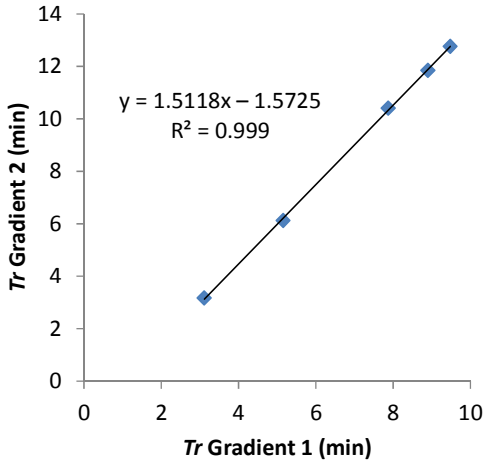
1.3. Retrospective analysis. Retention time re-calculation

It is important to appoint that in the retrospective analysis, the surface and wastewater samples had been analyzed in a previous study of Hernández et al. (2011) with different chromatography conditions, as shown in the table below.

Hence, in order to obtain comparable retention times (Tr), a gradient re-calculation was performed. For this purpose, the retention times of the five parent pharmaceuticals were measured in both gradient-conditions, *Gradient 1* (degradation experiments) and *Gradient 2* (Hernandez et al. (2011)) (See figure below). After performing a *Tr* graphical representation, a lineal equation was obtained ($y = 1.5118x - 1.5725$) where the correlation coefficient (r) was higher than 0.99. Finally, the equation was applied for obtaining the predicted retention time of TPs and metabolites in the gradient 2.

Gradient	Analytical column	Mobile phase	Percentage of organic modifier (B)	Flow rate
Gradient 1	Acquity UPLC BEH C18 (100x2.1 mm, 1.7 µm,Waters)	A: H_2O B: MeOH, both 0.01% (v/v) FA	0 min, 10%; 9 min, 90% 11 min, 90% 11.1 min, 10%; 14 min, 10%.	0.3 mL/min.
Gradient 2			0 min, 10%; 14 min,90%; 16 min, 90%; 16.01 min, 10% 18 min, 10%.	

	Tr gradient 1 (min)	*Tr* gradient 2 (min)
Ibuprofen	8.9	11.9
Gemfibrozil	9.5	12.8
Ofloxacin	3.1	3.2
Venlafaxine	5.2	6.1
Irbesartan	7.9	10.4



Compounds	*Tr* gradient 1 (min)	*Tr* gradient 2 (min)
Irbesartan	7.6	9.9
ISW1a	6.6	8.4
ISW1b	7.3	9.5
ISW2	7.3	9.5
IB3a	6.8	8.7
IB3b	7.1	9.2
IB4	6.4	8.1
IB5	6.6	8.4
IB6	6.3	8.0
Valsartan	7.9	10.4
Venlafaxine	5.2	6.3
VB1a	3.8	4.2
VB1b	5.2	6.3
VB2	5.1	6.1
VB3a	2.7	2.5
VB3b	3.1	3.1
VB4	3.9	4.3
V1	4.1	4.6
V2	4.0	4.5
Ofloxacin	3.1	3.1
OB1	3.8	4.2
Ibuprofen	9.0	12.0
IbSW1	6.6	8.4
IbSW2a	6.5	8.3
IbSW2b	8.1	10.7
IbSW2c	8.7	11.6
IbB3	6.2	7.8
IbB4	7.1	9.2
Ib1	6.9	8.9
Gemfibrozil	9.5	12.8
GSWB1	8.4	12.8

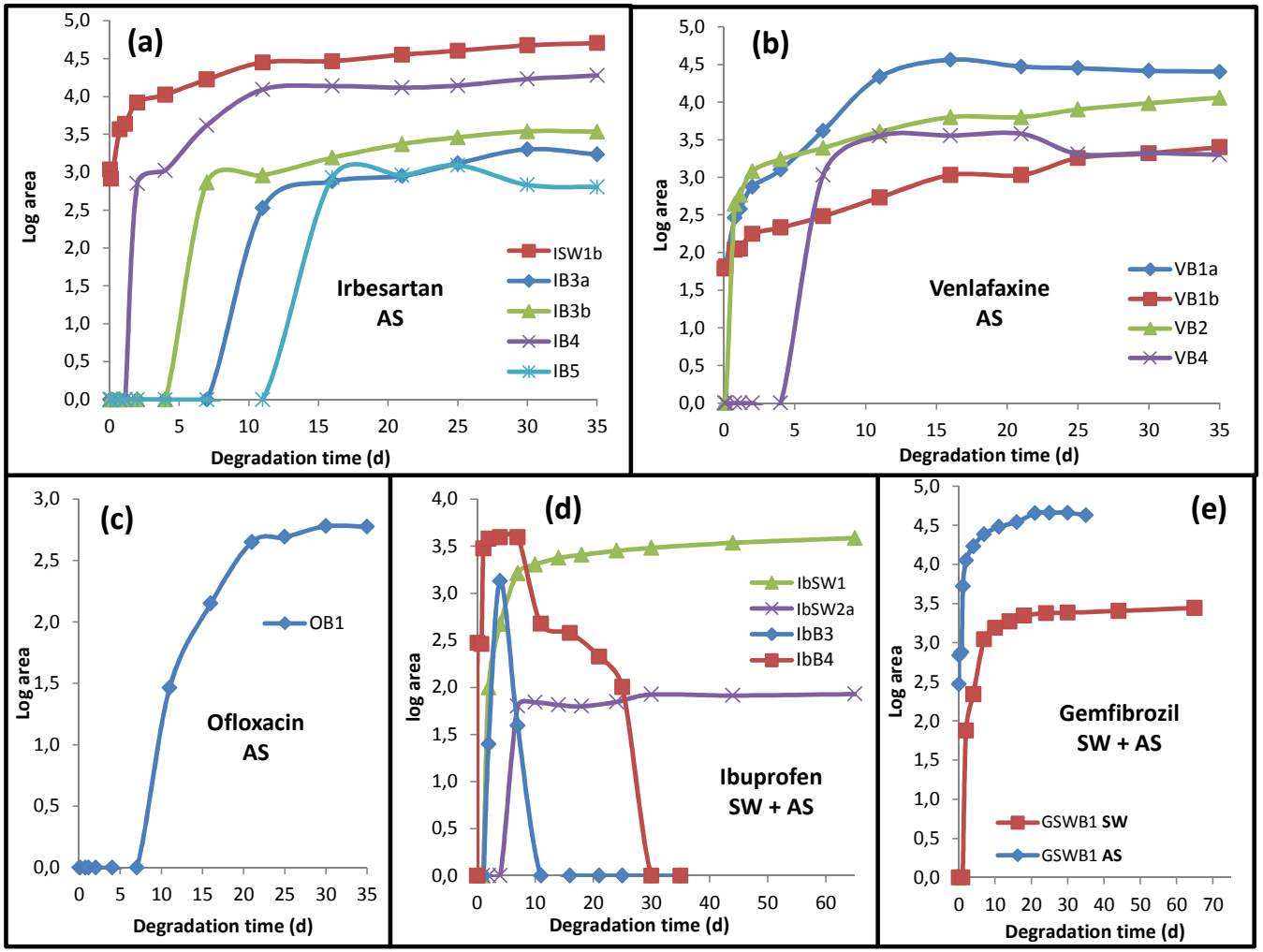


Figure 1SI. SemiLog-linear plot of formation of the main pharmaceutical TPs in: (a) irbesartan biodegradation in AS (b) venlafaxine in AS, (c) ofloxacin in AS, (d) ibuprofen biodegradation in SW and in AS and (e) gemfibrozil in SW and in AS.

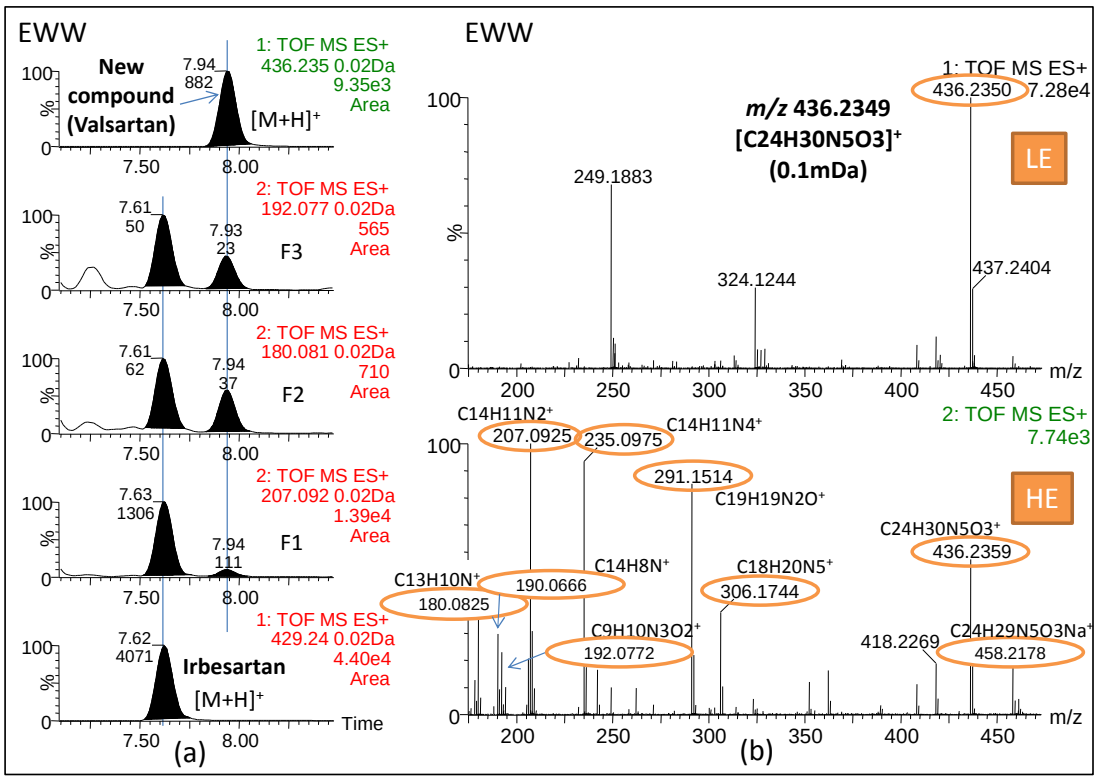


Figure 2SI. Detection of valsartan after applying the common fragmentation pathway strategy. (a) nw-XICs for $[M+H]^+$ of irbesartan and valsartan in LE, and three common fragment ions in HE. (b) LE and HE spectra of valsartan.

1.4. Proposed chemical structures and MS spectra for some TPs:

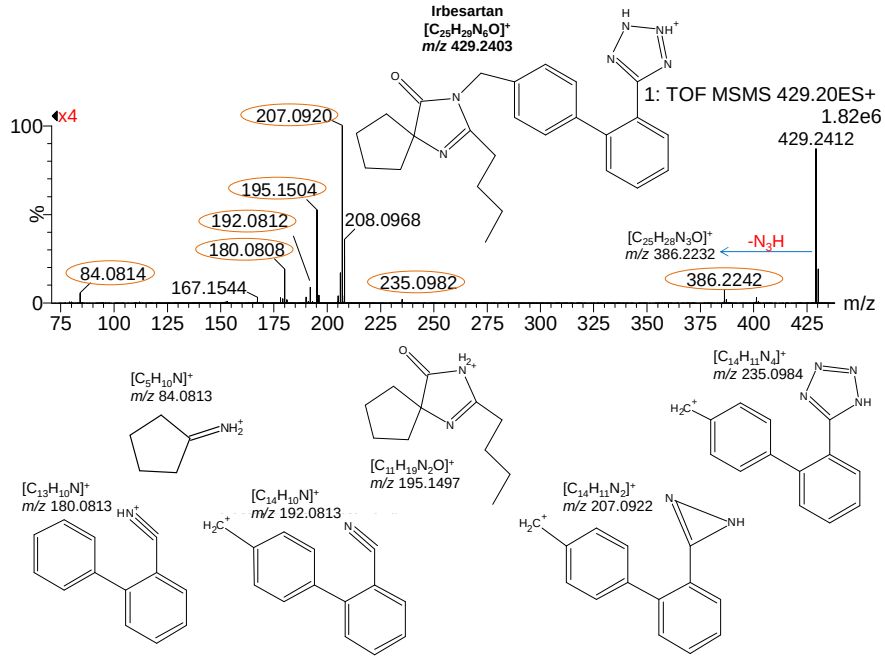


Figure 3SI. Fragmentation pathway of irbesartan.

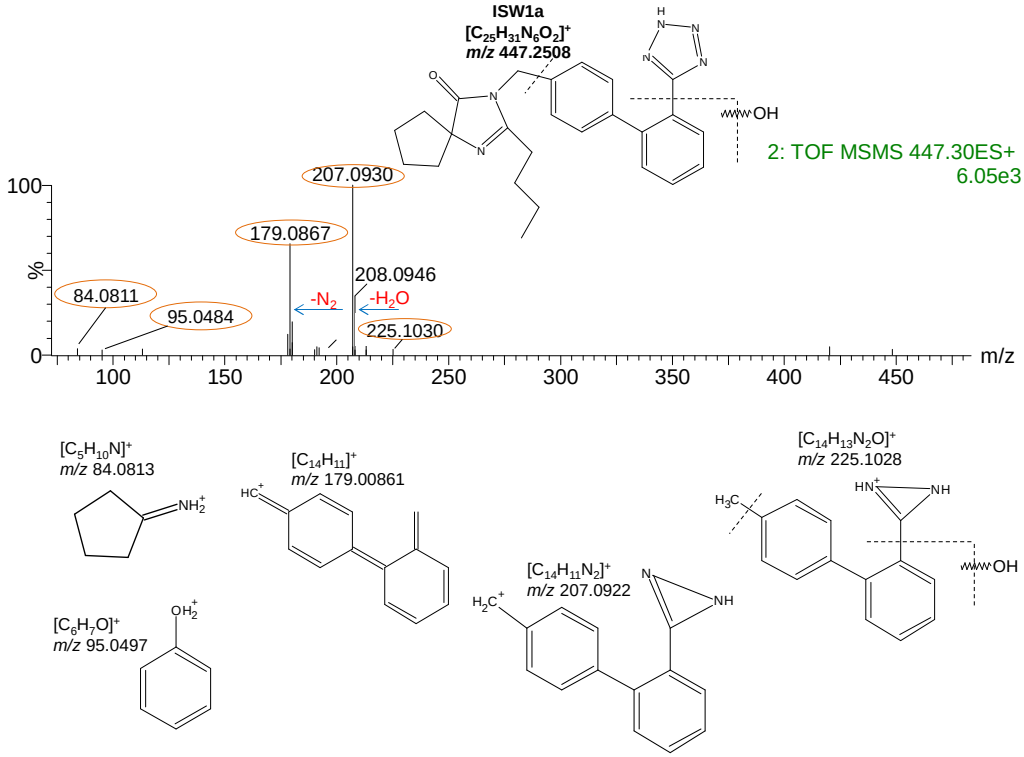


Figure 4SI. Elucidation of ISW1a.

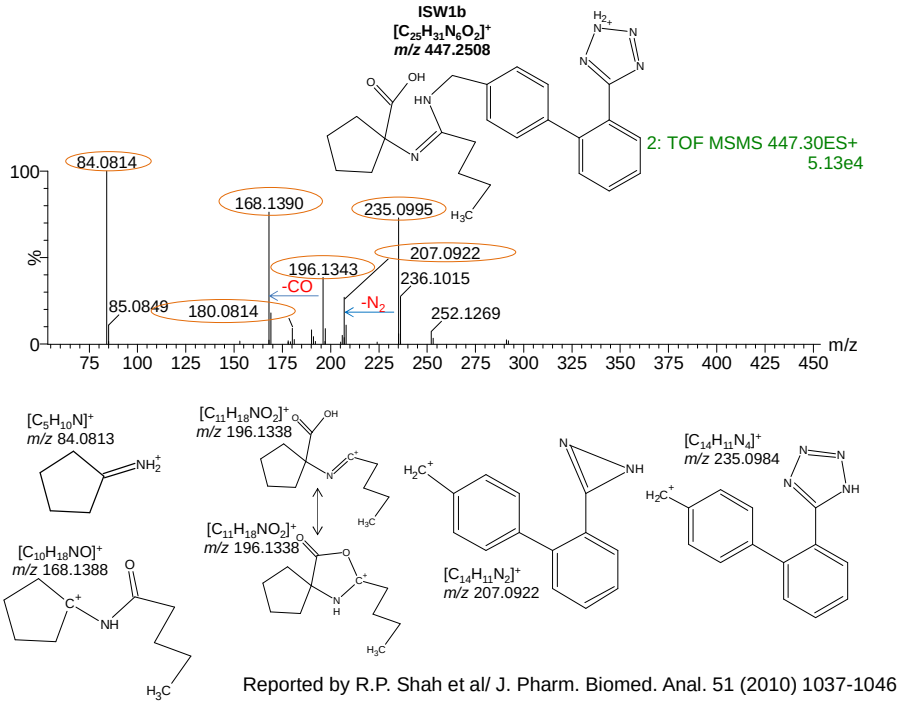


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Figure 5SI. Elucidation of ISW1b.

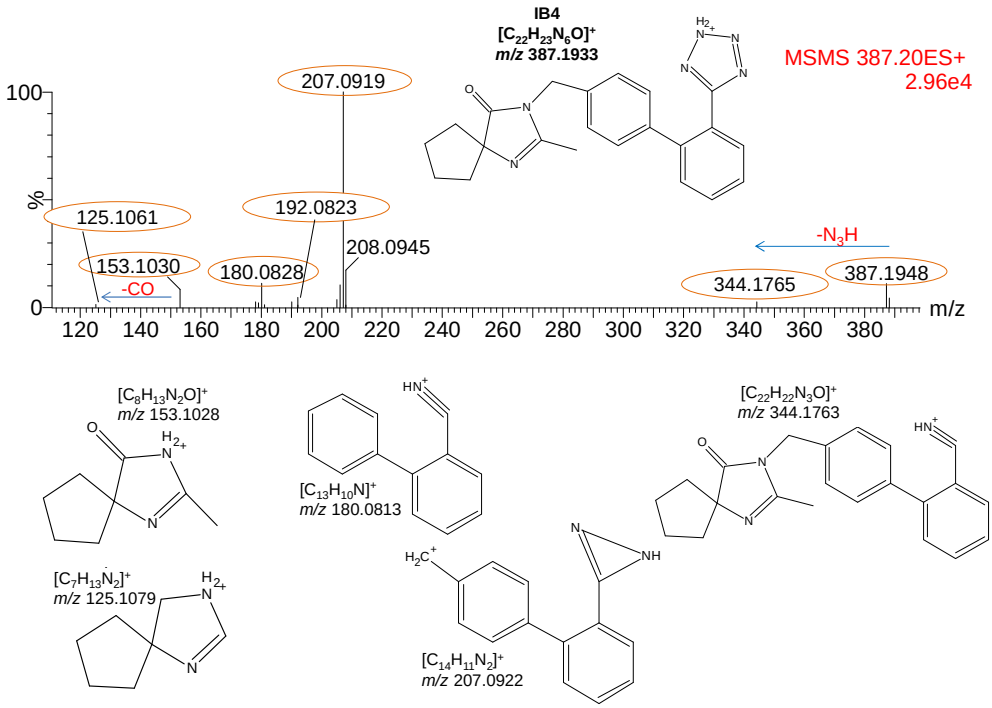

Figure 6SI. Elucidation of IB4.

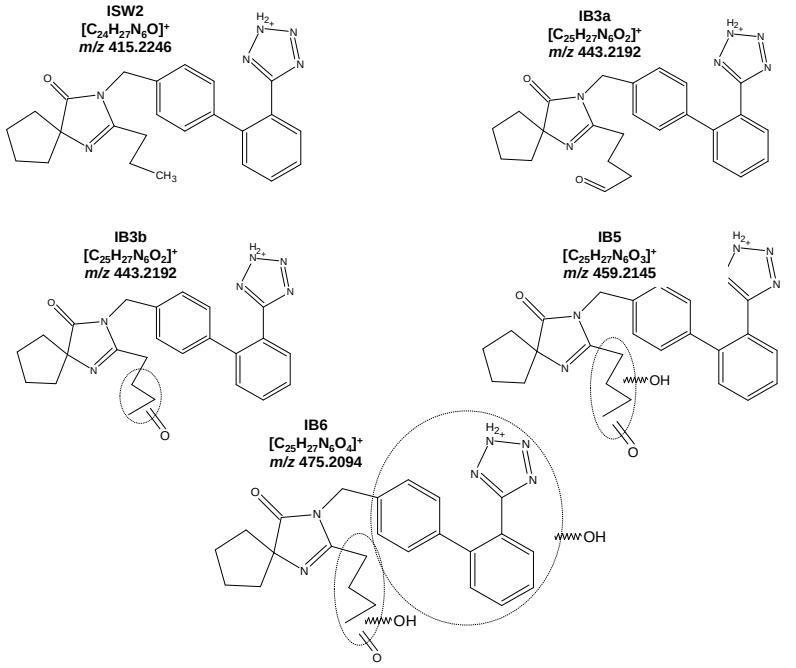

Figure 7SI. Elucidation of ISW2, IB3a, IB3b,IB5 and IB6.

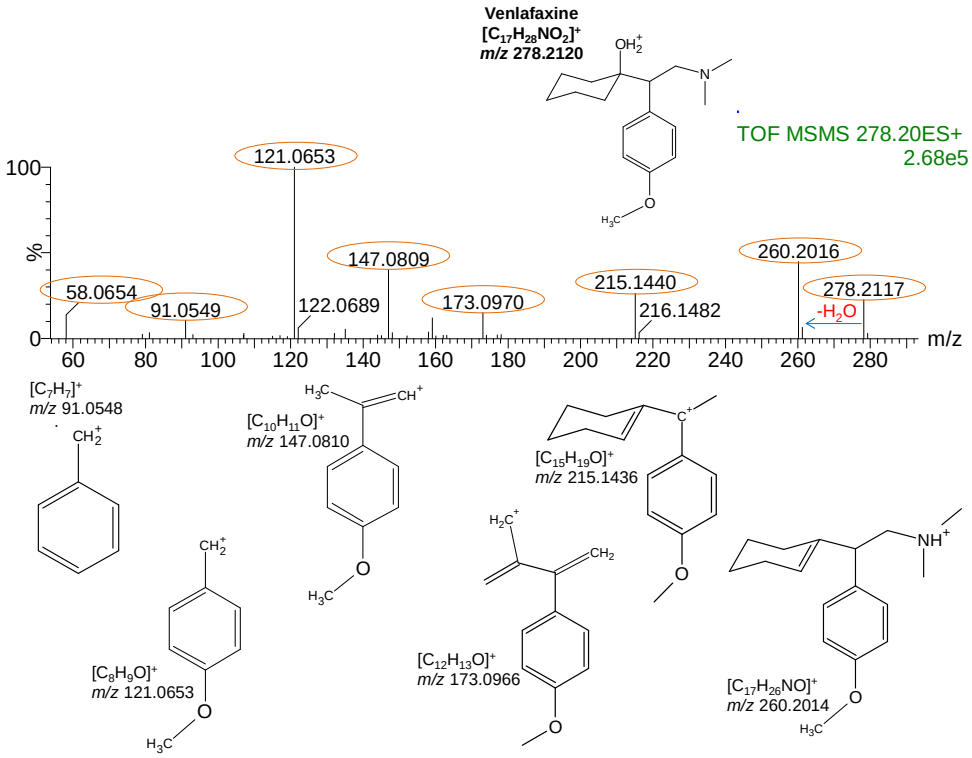

Figure 8SI. Fragmentation pathway of venlafaxine.

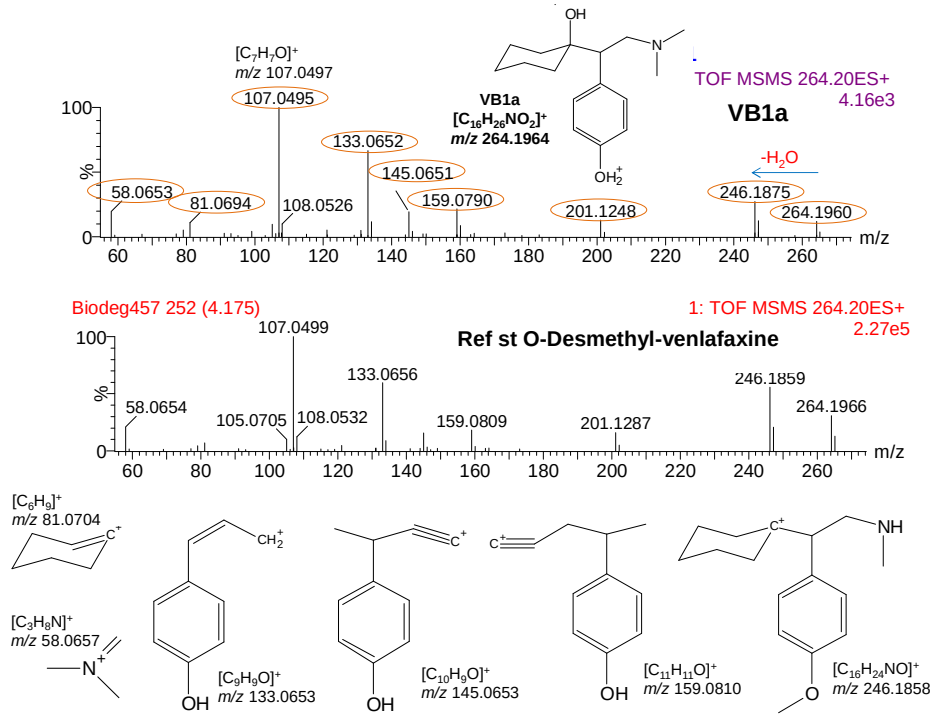

Figure 9SI. Elucidation of VB1a.

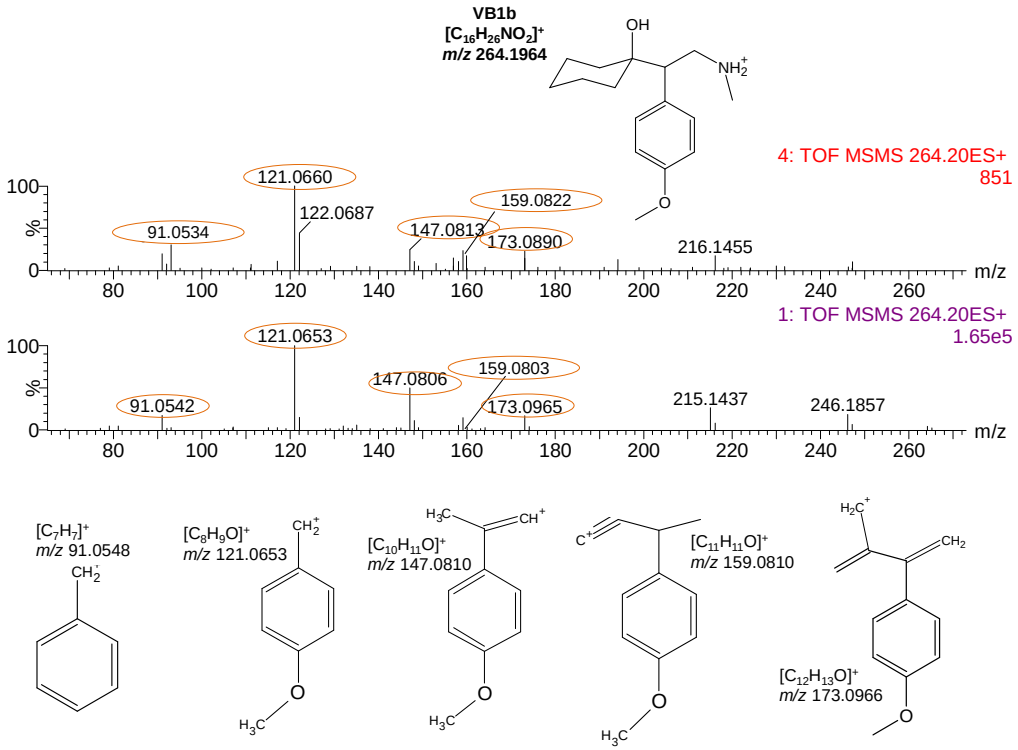

Figure 10SI. Elucidation of VB1b.

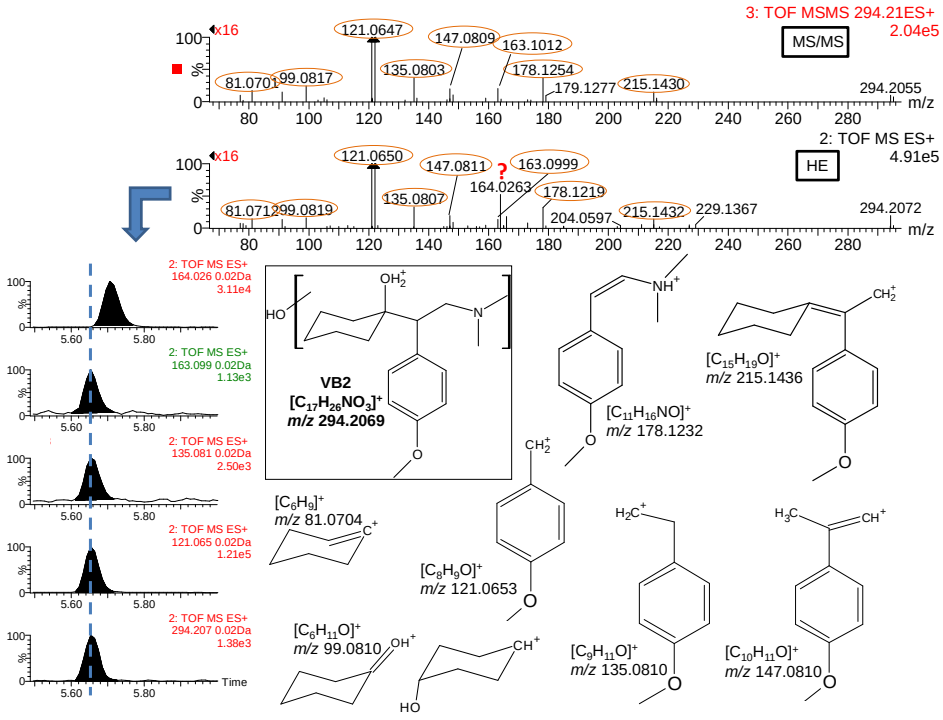

Figure 11SI. Elucidation of VB2.

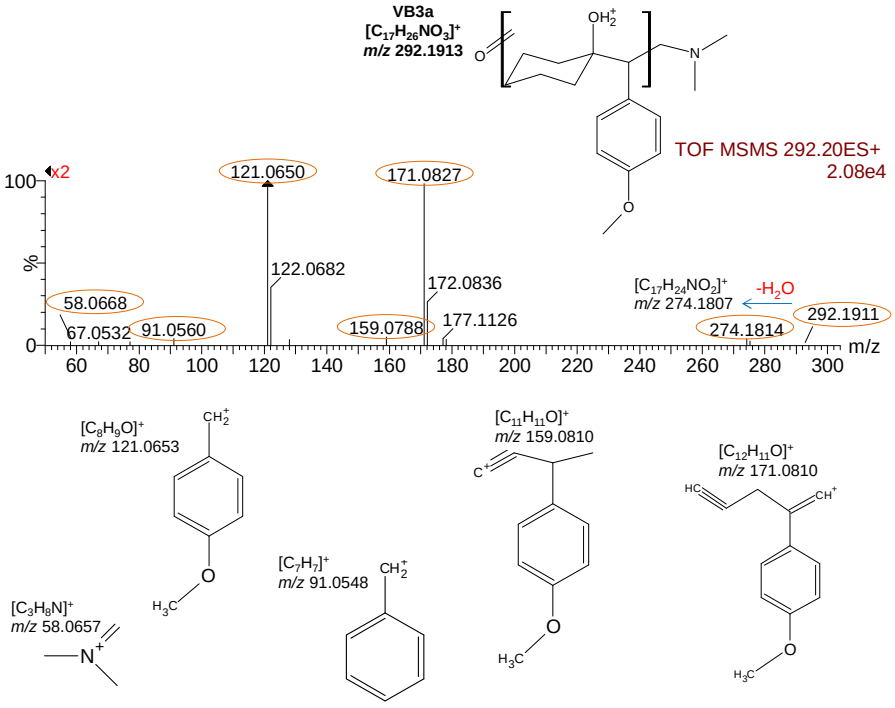

Figure 12SI. Elucidation of VB3a.

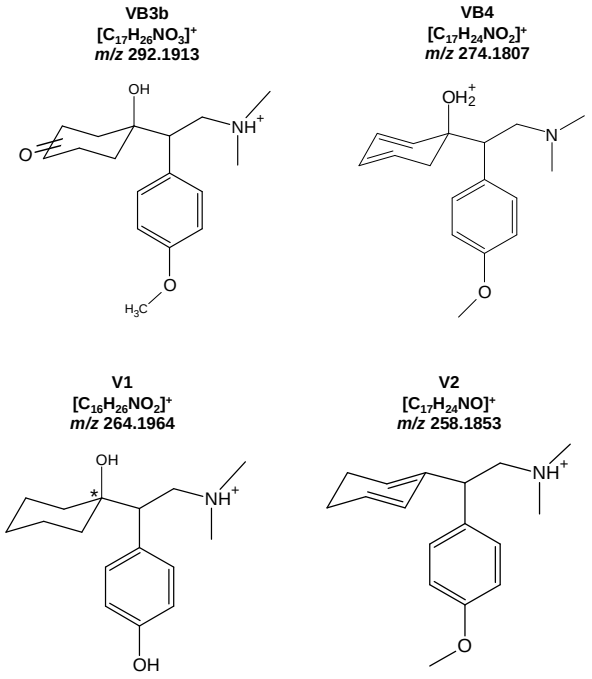

Figure 13SI. Elucidation of VB3b, VB4, V1 and V2.

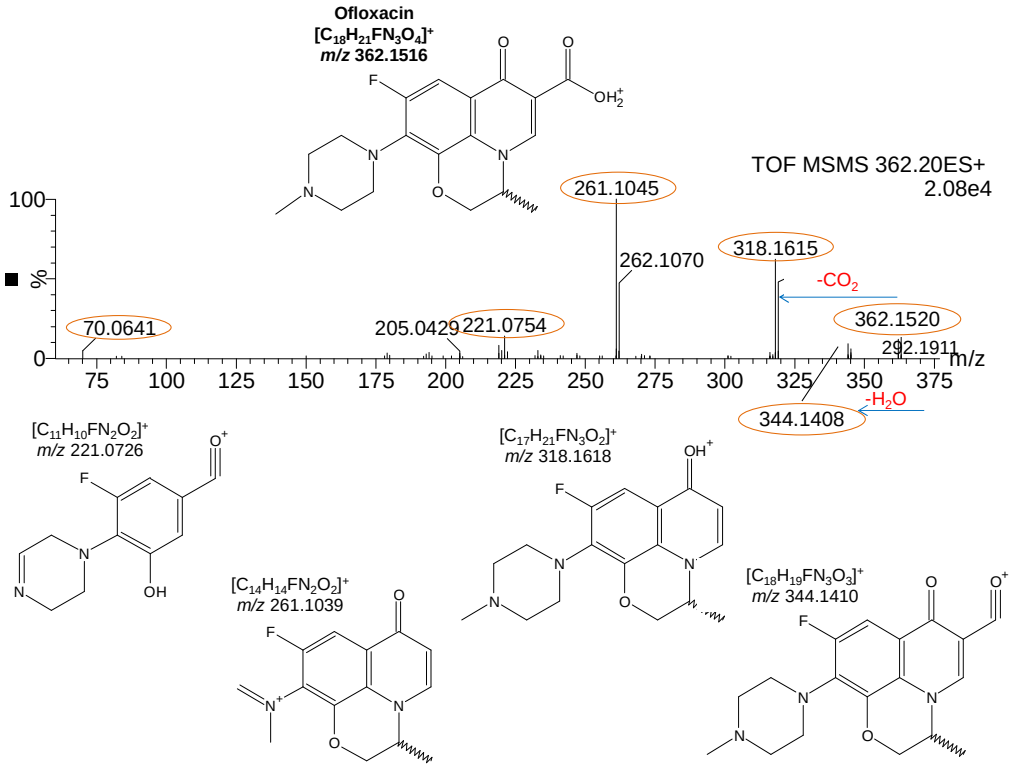

Figure 14SI. Fragmentation pathway of ofloxacin.

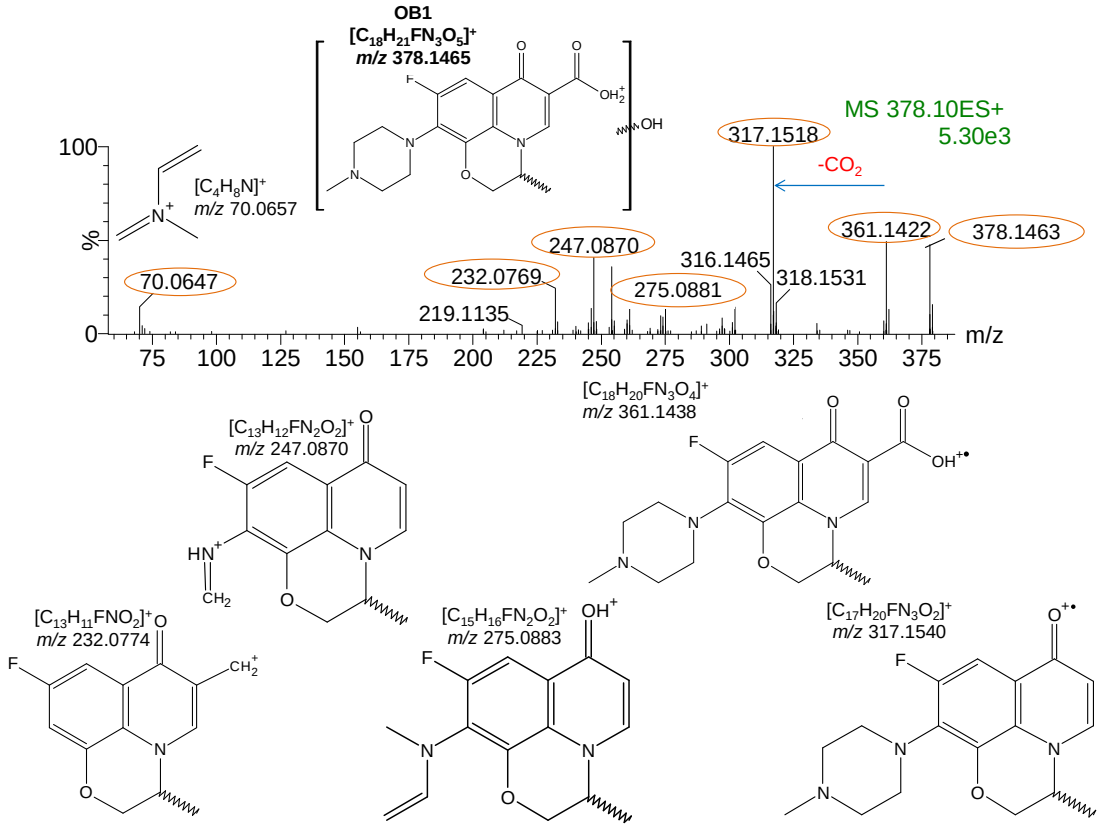

Figure 15SI. Elucidation of OB1.

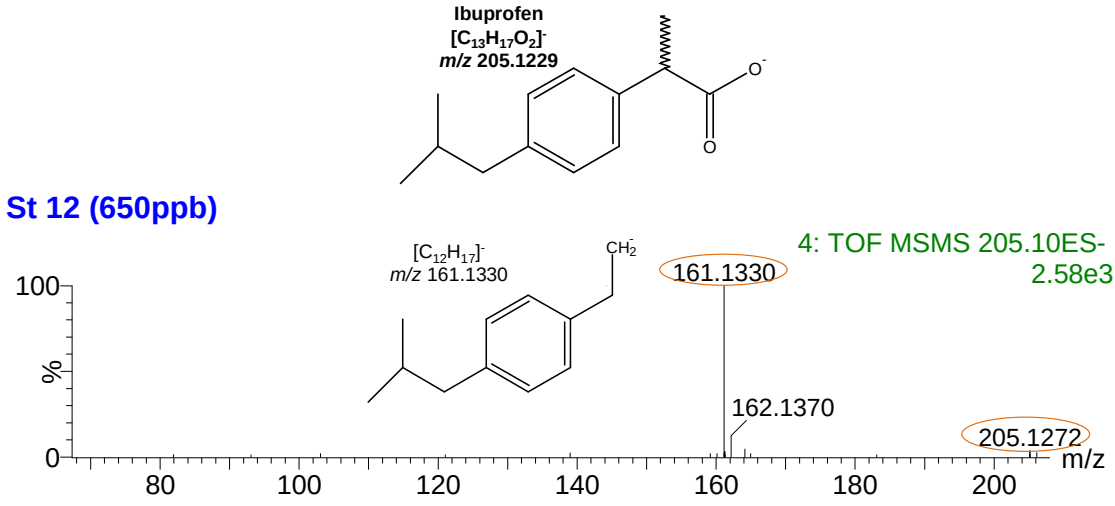

Figure 16SI. Fragmentation pathway of ibuprofen.

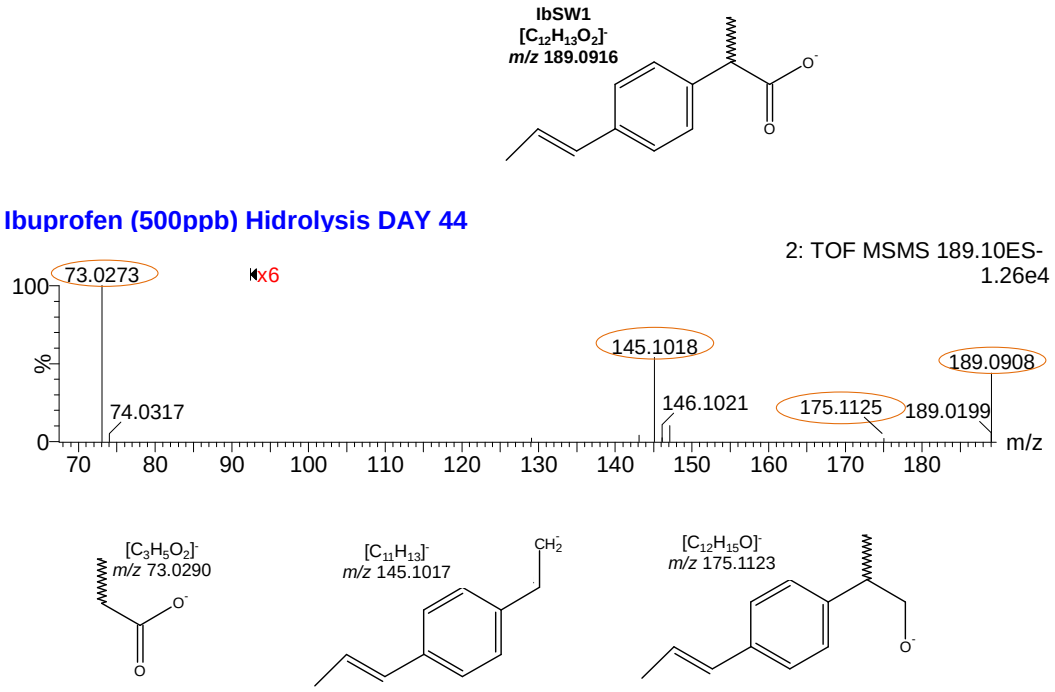

Figure 17SI. Elucidation of IbSW1.

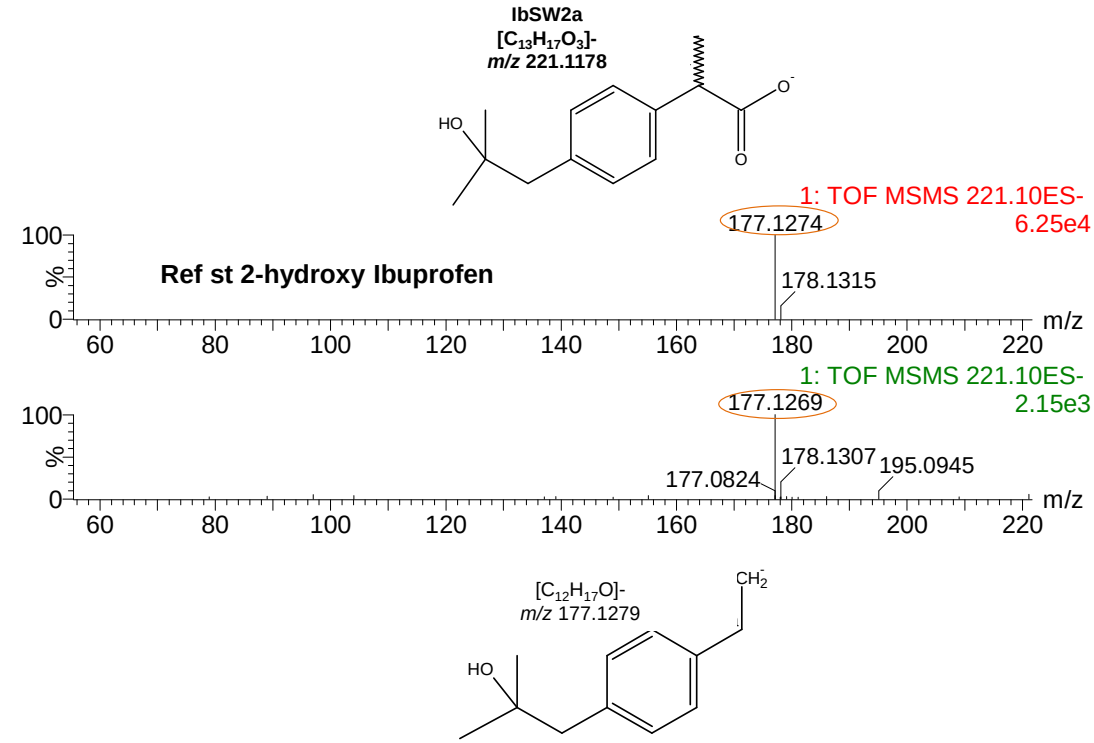

Figure 18SI. Elucidation of IbSW2a.

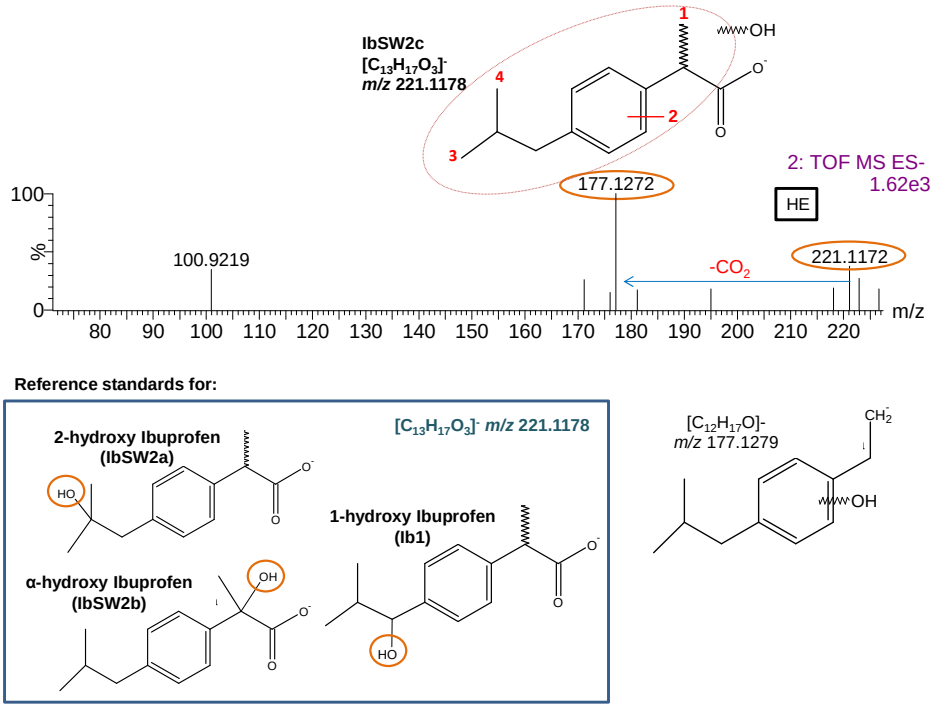

Figure 19SI. Elucidation of IbSW2c.

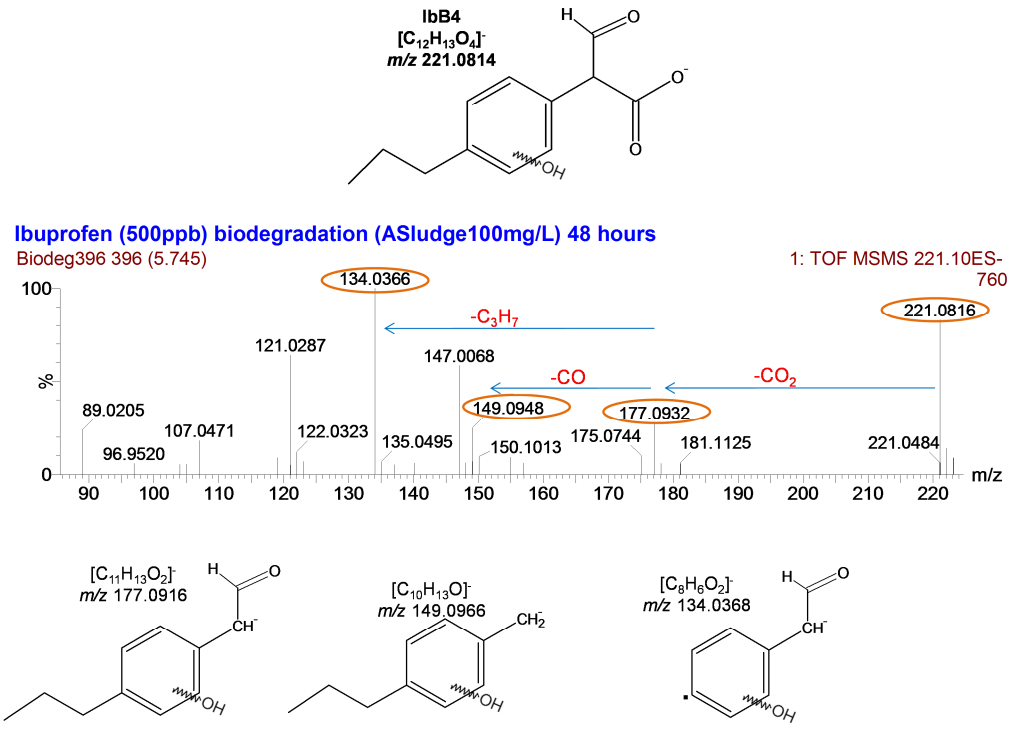

Figure 20SI. Elucidation of IbB4.

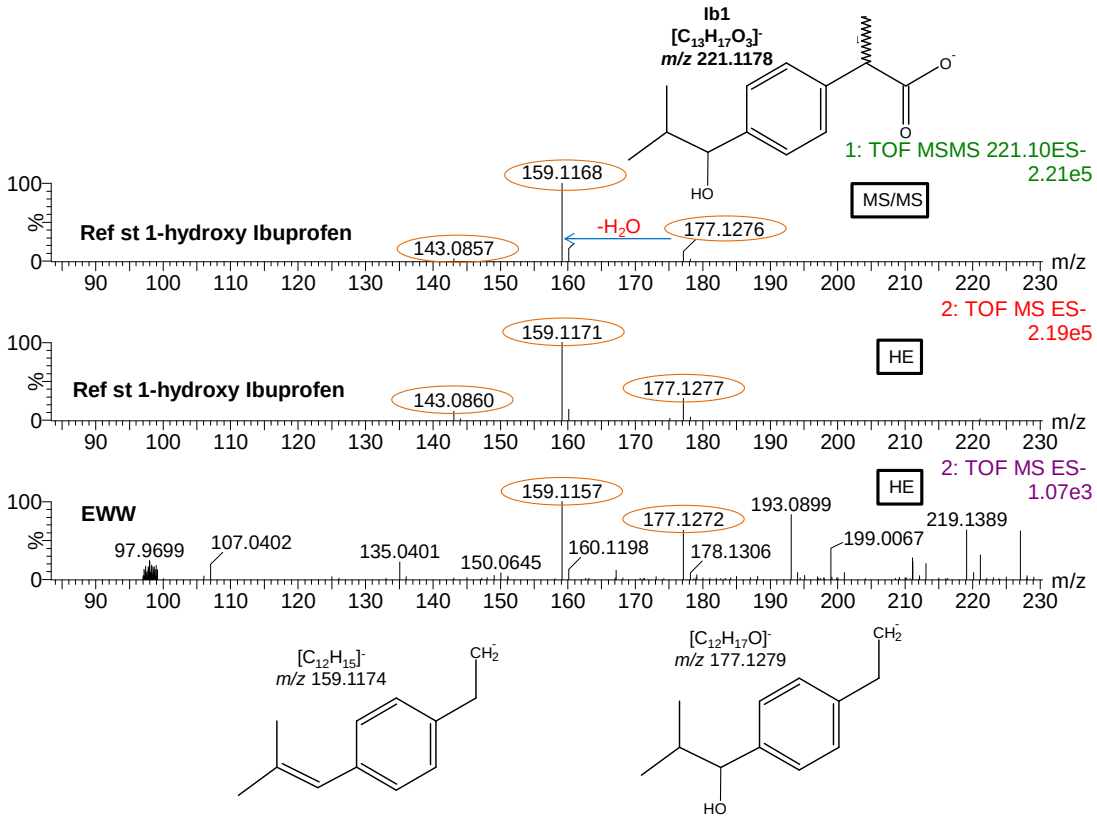

Figure 21SI. Elucidation of Ib1.

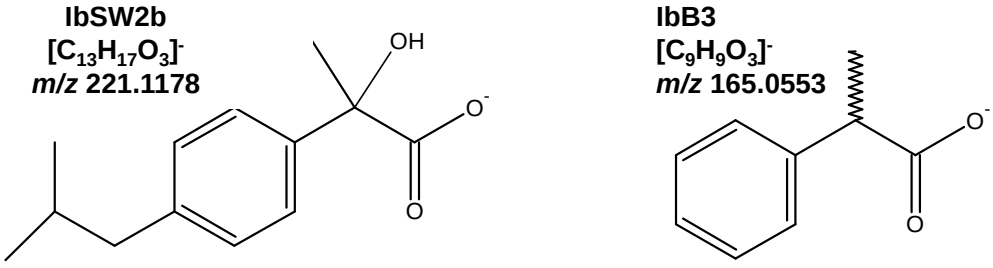

Figure 22SI. Elucidation of IbSW2b and IbB3.

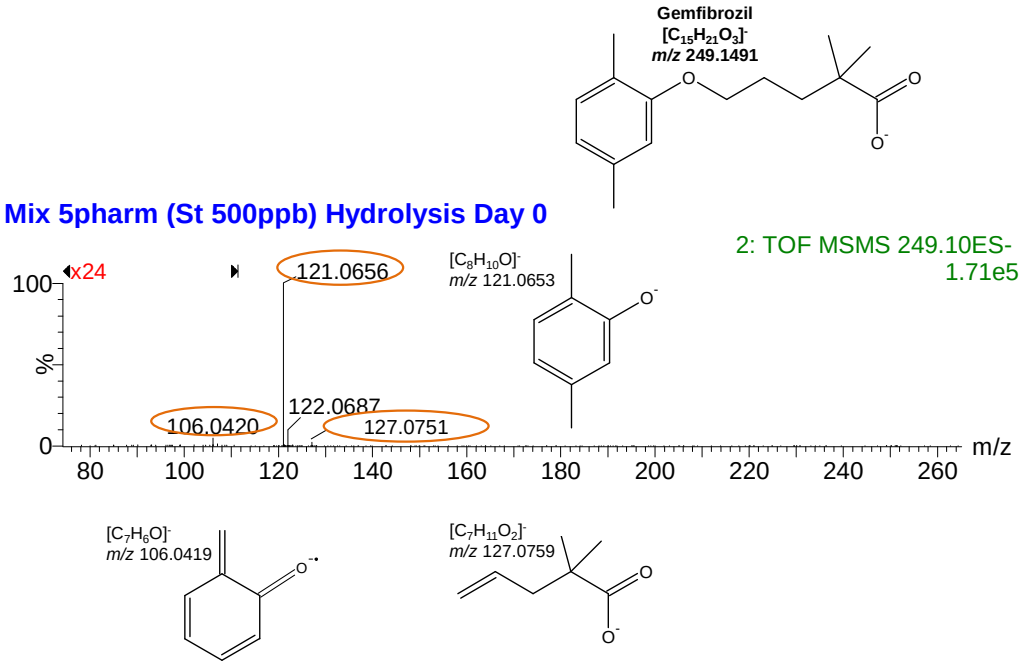

Figure 23SI. Fragmentation pathway of gemfibrozil.

Table 2SI. Irbesartan, valsartan and metabolites/TPs obtained in hydrolysis and biodegradation experiments by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Irbesartan	ESI+	7.6	C25H29N6O	429.2412	0.9	14.5	
			C14H11N2	207.0920	-0.2	10.5	
			C11H19N2O	195.1504	-0.7	3.5	
			C14H10N	192.0812	-0.1	10.5	
			C13H10N	180.0808	-0.5	9.5	
			C25H28N3O	386.2242	1.0	13.5	
			C5H10N	84.0814	-0.1	1.5	
			C14H11N4	235.0982	-0.2	11.5	
ISW1a	ESI+	6.6	C25H31N6O2	447.2493	-1.5	13.5	hydroxylation
			C14H11N2	207.0930	0.8	10.5	+hydrogenation
			C14H11	179.0867	0.6	9.5	
			C14H13N2O	225.1030	0.2	9.5	
			C6H7O	95.0484	-1.3	3.5	
			C5H10N	84.0811	-0.2	1.5	
ISW1b	ESI+	7.3	C25H31N6O2	447.2495	-1.3	13.5	hydroxylation
			C5H10N	84.0814	0.1	1.5	+hydrogenation
			C10H18NO	168.1390	0.2	2.5	
			C14H11N4	235.0995	1.1	11.5	
			C14H11N2	207.0922	0.0	10.5	
			C11H18NO2	196.1343	0.5	3.5	
			C13H10N	180.0814	-0.1	9.5	
			C14H10N	192.0810	-0.3	6.5	
			C14H8N	190.0659	0.2	11.5	
ISW2	ESI+	7.3	C24H27N6O	415.2236	-1.0	14.5	demethylation
			C14H11N2	207.0927	0.5	10.5	
			C24H26N3O	372.2056	-2.0	13.5	
			C5H10N	84.0811	-0.2	1.5	
			C10H17N2O	181.1349	0.8	3.5	
IB3a	ESI+	6.8	C25H27N6O2	443.2207	1.5	15.5	oxidation
			C14H11N2	207.0920	-0.2	10.5	
			C13H10N	180.0805	-0.8	9.5	
			C14H10N	192.0803	-1.0	6.5	
			C23H25N6O	401.2075	-1.5	14.5	
			C10H14NO	164.1085	1.0	4.5	
			C5H10N	84.0814	0.1	1.5	
IB3b	ESI+	7.1	C25H27N6O2	443.2205	1.3	15.5	oxidation
			C14H11N2	207.0914	-0.8	10.5	
			C14H11N4	235.0998	1.4	11.5	
			C13H10N	180.0808	-0.5	9.5	
			C14H10N	192.0803	-1.0	6.5	
			C8H13N2O	153.1008	-2.0	3.5	
			C5H8NO	98.0599	-0.7	2.5	
			C14H8N	190.0668	1.1	11.5	

Table 2SI (Cont). Irbesartan, valsartan and metabolites/TPs obtained in hydrolysis and biodegradation experiments by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass m/z	Mass error (mDa)	DBE	Transformation process
IB4	ESI+	6.4	C22H23N6O	387.1948	1.5	14.5	dealkylation (C_3H_7)
			C14H11N2	207.0919	-0.3	10.5	
			C13H10N	180.0828	1.5	9.5	
			C8H13N2O	153.1030	0.2	3.5	
			C14H10N	192.0826	1.0	10.5	
			C7H13N2	125.1061	-1.8	2.5	
			C14H11N4	235.0991	0.7	11.5	
			C5H10N	84.0815	0.2	1.5	
			C22H22N3O	344.1765	02	13.5	
			C14H8N	190.0669	1.2	11.5	
IB5	ESI+	6.6	C25H27N6O3	459.2137	-0.8	15.5	hydroxylation +oxidation
			C14H11N2	207.0924	0.2	10.5	
			C14H13N2O	225.1043	1.5	9.5	
			C14H11N4	235.0991	0.7	11.5	
			C13H10N	180.0824	1.1	9.5	
			C14H8N	190.0667	1.0	11.5	
IB6	ESI+	6.3	C25H27N6O4	475.2096	0.2	15.5	2 hydroxylation +oxidation
			C14H11N2	207.0926	0.4	10.5	
			C14H13N2O	225.1032	0.4	9.5	
			C8H13N2O	153.1060	3.2	3.5	
			C14H11N4	235.0994	1.0	11.5	
			C24H24N5O3	430.1890	1.1	15.5	
			C13H10N	180.0820	0.7	9.5	
			C8H13N2	137.1073	-0.6	3.5	
			C14H9O	193.0670	1.7	10.5	
			C14H8N	190.0663	0.6	11.5	
Valsartan	ESI+	7.9	C24H30N5O3	436.2350	0.1	12.5	
			C14H11N2	207.0925	0.3	10.5	
			C14H11N4	235.0975	-0.9	11.5	
			C19H19N2O	291.1514	1.7	11.5	
			C13H10N	180.0825	1.2	9.5	
			C9H10N3O2	192.0772	-0.1	6.5	
			C14H8N	190.0648	-0.9	11.5	
			C18H20N5	306.1737	1.8	11.5	
			C24H29N5O3Na	458.2178	1.0	12.5	

Table 3SI. Venlafaxine and metabolites/TPs obtained by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process*
Venlafaxine	ESI+	5.2	C17H28NO2	278.2117	-0.3	4.5	
			C17H26NO	260.2016	0.2	5.5	
			C15H19O	215.1440	0.4	6.6	
			C10H11O	147.0809	-0.1	5.5	
			C7H7	91.0549	0.1	4.5	
			C12H13O	173.0970	0.4	6.5	
			C3H8N	58.0654	-0.3	0.5	
			C8H9O	121.0653	-0.0	4.5	
VB1a O-Desmethyl-venlafaxine	ESI+	3.8	C16H26NO2	264.1960	-0.4	4.5	demethylation
			C7H7O	107.0495	-0.2	4.5	
			C11H11O	159.0790	-2.0	6.5	
			C3H8N	58.0653	-0.4	0.5	
			C6H9	81.0694	-1.0	2.5	
			C14H17O	201.1248	-3.1	6.5	
			C9H9O	133.0652	-0.1	5.5	
			C16H24NO	246.1875	1.7	5.5	
			C10H9O	145.0651	-0.2	6.5	
			C8H9O	121.0647	-0.6	4.5	
VB1b N-Desmethyl-venlafaxine	ESI+	5.2	C16H26NO2	264.1963	-0.1	4.5	demethylation
			C8H9O	121.0660	0.7	4.5	
			C10H11O	147.0813	0.3	5.5	
			C16H24NO	246.1857	-0.1	5.5	
			C12H13O	173.0965	-0.1	6.5	
			C15H19O	215.1437	0.1	6.5	
			C11H11O	159.0822	1.2	6.5	
			C7H7	91.0534	-1.4	4.5	
VB2	ESI+	5.1	C17H28NO3	294.2055	-1.4	4.5	hydroxylation
			C8H9O	121.0647	-0.6	4.5	
			C9H11O	135.0803	-0.7	4.5	
			C10H11O	147.0809	-0.1	5.5	
			C11H16NO	178.1254	2.2	4.5	
			C6H11O	99.0817	0.7	1.5	
			C6H9	81.0701	-0.3	2.5	
			C15H19O	215.1430	-0.6	6.5	
VB3a	ESI+	2.7	C17H26NO3	292.1911	-0.2	5.5	oxidation
			C17H24NO2	274.1814	0.7	6.5	
			C8H9O	121.0650	-0.3	4.5	
			C12H11O	171.0827	1.7	7.5	
			C11H11O	159.0788	-2.2	6.5	
			C7H7	91.0560	1.2	4.5	
			C3H8N	58.0668	-1.1	0.5	
VB3b	ESI+	3.1	C17H26NO3	292.1913	0.0	5.5	oxidation
			C17H24NO2	274.1817	1.0	6.5	
			C8H9O	121.0655	0.2	4.5	
			C8H11O2	139.0769	1.0	3.5	
			C8H14NO2	156.1029	0.4	2.5	
			C7H12N	110.0968	-0.2	2.5	

Table 3SI (Cont). Venlafaxine and metabolites/TPs obtained by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass m/z	Mass error (mDa)	DBE	Transformation process*
VB4	ESI+	3.9	C17H24NO2	274.1834	2.7	6.5	2*dehydrogenation
			C8H9O	121.0652	-0.1	4.5	
			C12H11O	171.0795	-1.5	7.5	
			C14H11	179.0854	-0.7	9.5	
			C15H15O	211.1110	-1.3	8.5	
			C14H12O	196.0878	-1.0	9.0	
			C11H11O	159.0808	-0.2	6.5	
			C12H9	153.0722	1.8	8.5	
			C9H9O	133.0668	1.5	5.5	
			C7H9	93.0705	0.1	3.5	
V1	ESI+	4.1	C16H26NO2	264.1974	1.0	4.5	demethylation
			C3H8N	58.0660	0.3	0.5	
			C16H24NO	246.1861	0.3	5.5	
			C8H11O2	139.0773	1.4	3.5	
			C7H9	93.0722	1.8	3.5	
			C14H15O	199.1134	1.1	7.5	
			C14H17O	201.1294	1.5	6.5	
			C11H9O	157.0670	1.7	7.5	
V2	ESI+	4.0	C17H24NO	258.1869	1.1	6.5	oxidation+
			C14H15O	199.1130	1.1	7.5	2*dehydrogenation
			C14H17O	201.1290	0.7	6.5	
			C11H9O	157.0664	1.1	7.5	
			C9H9O	133.0672	1.9	5.5	

Table 4SI. Ofloxacin and the TP obtained in biodegradation experiments by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Ofloxacin	ESI+	3.1	C18H21N3O4F	362.1520	-0.4	9.5	
			C17H21N3O2F	318.1615	-0.3	8.5	
			C14H14N2O2F	261.1045	0.6	8.5	
			C18H19N3O3F	344.1408	-0.2	10.5	
			C11H10N2O2F	221.0754	2.8	7.5	
			C4H8N	70.0641	-1.6	1.5	
OB1	ESI+	3.8	C18H21N3O5F	378.1463	-0.2	9.5	hydroxylation
			C17H20N3O2F	317.1518	-2.2	9.0	
			C13H12N2O2F	247.0870	-1.3	8.5	
			C18H20N3O4F	361.1422	-1.63	10.0	
			C12H15N2O3F	254.1062	-0.5	6.0	
			C13H11NO2F	232.0769	-0.5	8.5	
			C16H17N3O2F	302.1325	2.0	9.5	
			C4H8N	70.0647	-1.0	1.5	
			C15H16N2O2F	275.1210	1.4	8.5	

Table 5SI. Ibuprofen and TPs obtained in hydrolysis and biodegradation experiments by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Ibuprofen	ESI-	9.0	C13H17O2	205.1272	4.3	5.5	
			C12H17	161.1330	0.0	4.5	
IbSW1	ESI-	6.6	C12H13O2	189.0908	-0.8	6.5	demethylation+
			C11H13	145.1018	0.1	5.5	dehydrogenation
			C3H5O2	73.0273	-1.7	1.5	
			C12H15O	175.1125	0.2	5.5	
IbSW2a.b.c 2-hydroxy ibuprofen (IbSW2a) α-hydroxy ibuprofen (IbSW2b)	ESI-	6.5-8.1-8.7	C13H17O3	221.1182	0.4	5.5	hydroxylation
			C12H17O	177.1274	-0.5	4.5	
IbB3	ESI-	6.2	C9H9O3	165.0545	-0.7	5.5	dealkylation (C_4H_9)+ hydroxylation
IbB4	ESI-	7.1	C12H13O4	221.0816	0.2	6.5	demethylation
			C11H13O2	177.0932	1.6	5.5	+O hydroxylation
			C10H13O	149.0948	-1.8	4.5	
			C8H6O2	134.0366	-0.2	6.0	
Ib1 1-hydroxy ibuprofen	ESI-	6.9	C13H17O3	221.1180	0.2	5.5	hydroxylation
			C12H17O	177.1272	-0.7	4.5	
			C12H15	159.1157	-1.7	5.5	
			C11H11	143.0857	-0.4	6.5	

Table 6SI. Gemfibrozil and TP obtained in hydrolysis and biodegradation experiments by LC-ESI-QTOF MS.

Compound	Ionization mode	Ret time (min)	Elemental composition	Accurate mass *m/z*	Mass error (mDa)	DBE	Transformation process
Gemfibrozil	ESI-	9.5	C15H21O3	249.1486	-0.5	5.5	
			C8H9O	121.0656	0.3	4.5	
			C15H20NaO3	271.1328	1.8	5.5	
			C7H11O2	127.0751	-0.8	2.5	
			C7H6O	106.0420	0.1	5.0	
GSWB1	ESI-	8.4	C15H19O5	279.1241	0.9	6.5	hydroxylation
			C15H18O5Na	301.1053	0.1	6.5	+ oxidation
			C7H7O	107.0500	0.3	4.5	
			C14H18O3Na	257.1158	0.4	5.5	
			C8H7O3	151.0396	0.1	5.5	
			C13H17O	189.1280	0.1	5.5	
			C8H6O3Na	173.0213	-0.2	5.5	

5.3 Discusión de los resultados

Una vez realizados los experimentos de biodegradación y los análisis mediante UHPLC-QTOF MS, los datos se procesaron utilizando el software específico MetaboLynx XS™. A modo de ejemplo, la **Figura 5.1** muestra una captura de pantalla de la detección de un TP de biodegradación del gemfibrozil siguiendo la estrategia analítica aplicada en este trabajo.

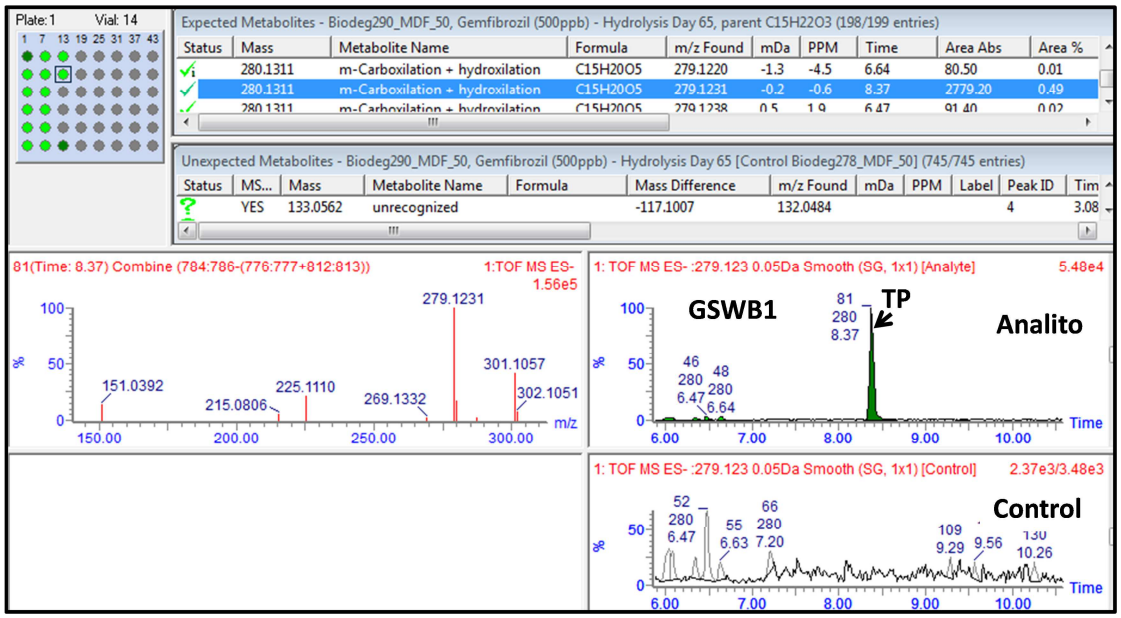

Figura 5.1 Detección de un TP de biodegradación del gemfibrozil mediante el software MetaboLynx XS™.

Para el irbesartán se detectaron 3 y 5 TPs en los experimentos en agua superficial y en lodos activados, respectivamente. Por lo que se refiere a venlafaxina y ofloxacino se generaron 6 y 1 TPs, respectivamente, únicamente en los experimentos con lodos. En todos estos casos, tanto los fármacos como los TPs fueron observados en modo positivo de ionización (ESI+). Por otro lado, el ibuprofeno, gemfibrozil y sus TPs se detectaron en modo negativo (ESI-). El ibuprofeno generó 4 y 2 TPs en los experimentos en agua superficial y en lodos activados, respectivamente. Por último,

el gemfibrozil fue completamente degradado para dar un único TP tras la aplicación de lodos activados. Cabe decir que dicho TP ya había sido detectado, aunque a considerable menor concentración, en los experimentos llevados a cabo en aguas superficiales **(Tabla 5.1).**

Tabla 5.1 Productos de transformación detectados mediante las dos estrategias aplicadas en este trabajo para la búsqueda de TPs/metabolitos.

TPs detectados	MetaboLynx XS		Iones fragmentos comunes
Biodegradación	Agua superficial	Lodos activados	
Irbesartán	3	5	1
Venlafaxina	-	6	2
Ofloxacino	-	1	-
Ibuprofeno	4	2	1
Gemfibrozil*	1	1	-

* En el caso del gemfibrozil, se detectó el mismo TP en agua superficial y en lodos activados.

Como muestran las *Tablas 2-6SI*, *Artículo Científico 6*, a todos los TPs se les asignó una fórmula molecular y un tiempo de retención. Además, se intentaron elucidar, realizando una identificación tentativa. Para ello, se estudiaron los espectros a masa exacta, obtenidos a baja y a alta energía de colisión, así como los espectros de MS/MS que se obtuvieron en análisis adicionales para los TPs más abundantes.

Por otro lado, se detectaron 4 compuestos tras aplicar la metodología basada en la búsqueda de iones fragmento comunes (**Tabla 5.1**) en aguas "reales" de efluente y superficial. Entre ellos se identificó el valsartán (*Figura 2SI, Artículo Científico 6*), otro fármaco de la misma familia que el irbesartán, muy similar en su estructura. También se detectaron 2 productos de transformación de la venlafaxina (*Figura 3, Artículo Científico 6*) y uno más del ibuprofeno. Todos ellos se intentaron elucidar aprovechando la información obtenida del QTOF MS. Sus características están resumidas en las *Tablas 2SI ,3SI* y *5SI* del *Artículo Científico 6*.

Una vez identificados de forma tentativa los productos de transformación, el siguiente paso fue investigar su presencia en muestras reales. Para ello, se llevó a cabo un análisis retrospectivo en 38 muestras de efluente y 18 aguas superficiales, utilizando el software ChromaLynx XS™.

La **Figura 5.2** muestra la detección del gemfibrozil y de su TP GSWB1 en una muestra de efluente tras el análisis retrospectivo, utilizando el ChromaLynx XS.

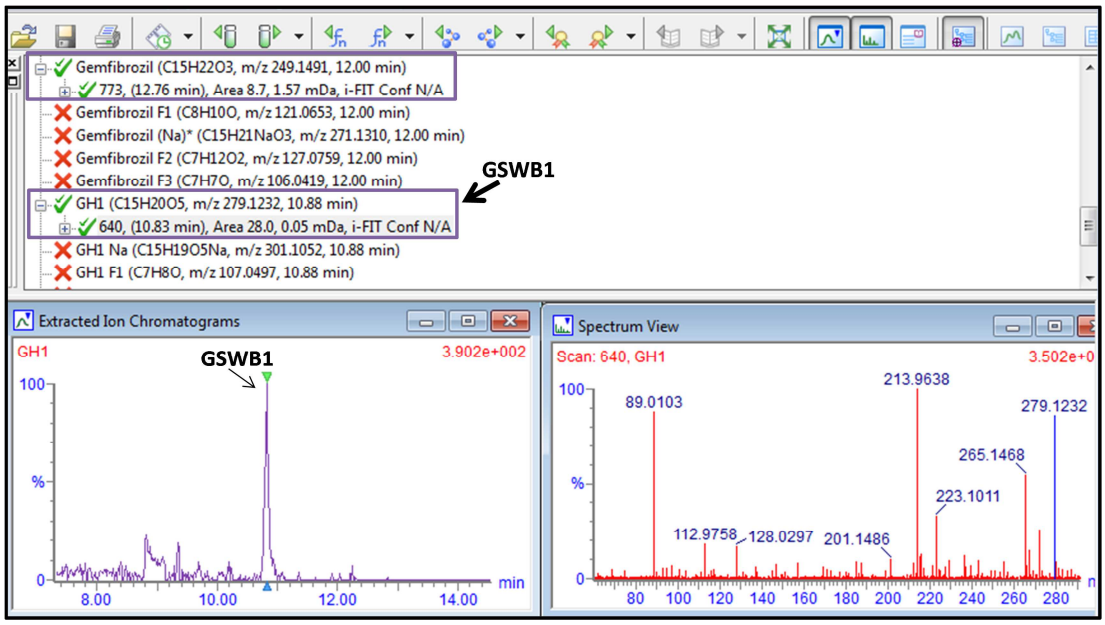

Figura 5.2 Detección del gemfibrozil y su TP GSWB1 en EWW mediante el ChromaLynx XS™.

La *Tabla 1* del *Artículo Científico 6* muestra un resumen de los positivos encontrados. Se detectaron hasta 19 compuestos en muestras de efluente, incluyendo los fármacos inalterados y algunos de sus TPs descubiertos. Entre ellos, cabe destacar el irbesartán (detectado en el 92% de EWW y 39% de SW), 5 de sus TPs (frecuencias de detección entre 32-87% en EWW y 6-22% en SW) y el valsartán (79% en EWW y 33% en SW). La venlafaxina también estuvo presente en un 87% de las muestras de efluente analizadas (22% en SW); además sus TPs (VB1a, VB1b y V2) se detectaron

con igual o mayor frecuencia estando presentes en un 92, 92, y 87% de los efluentes analizados, respectivamente. El ofloxacino se observó en los efluentes (82%) y en las aguas superficiales (17%); no obstante su TP no se detectó en ninguna de las muestras. Por lo que respecta al ibuprofeno, se detectó tanto en efluente (11%) como en muestras superficiales (6%), pero en ambos casos con una frecuencia inferior que la de sus TPs IbSW2a (16% en EWW y 11% en SW), IbB4 (34% en EWW y 50% en SW) y Ib1 (21% en EWW y 6% en SW). Un comportamiento similar se observó también para el gemfibrozil (24% en EWW y 22% en SW) y su TP, GSWB1 (71% en EWW y 33% en SW).

En total se encontraron hasta 13 TPs en las muestras. Estos compuestos, especialmente los detectados con más frecuencia que su *parent*, deberían ser candidatos para futuros métodos multi-residuos aplicados en el control de fármacos en aguas. De este modo, y no sólo investigando los compuestos inalterados, se tendría una visión más realista sobre qué compuestos emergentes están presentes en el medio ambiente y cómo influyen en él.

Sin embargo, el primer paso debería ser la adquisición de patrones de referencia de los TPs para confirmar, de una manera inequívoca, su identidad. Así pues, se adquirieron patrones de referencia para aquellos TPs detectados en muestras reales, en los casos en los que estaban comercialmente disponibles: O-desmetil venlafaxina, N-desmetil venlafaxina, 1-hidroxi ibuprofeno, 2-hidroxi ibuprofeno y α-hidroxi ibuprofeno.

Tras inyectarlos en el LC-QTOF MS se pudo confirmar la identidad de los 5 TPs tentativamente identificados, lo que demuestra la utilidad de la información aportada por el QTOF MS en los procesos de elucidación estructural:

- O-desmetil venlafaxina → VB1a

- N-desmetil venlafaxina → VB1b
- 1-hidroxi ibuprofeno → Ib1
- 2-hidroxi ibuprofeno → IbSW2a
- α-hidroxi ibuprofeno → IbSW2b

A modo de ejemplo, la **Figura 5.3** muestra los cromatogramas nw-XICs, con una ventana de masa de ±0.02 Da, de los patrones adquiridos, observándose que eluyen al mismo tiempo de retención que en las muestras sometidas a ensayos de degradación, o en el caso del Ib1, en la muestra de efluente urbano. Además de compartir el mismo tiempo de retención (tolerancia máxima ±2.5%), un requisito indispensable para la confirmación fue compartir el *m/z* de la molécula (des)protonada y como mínimo un ion fragmento.

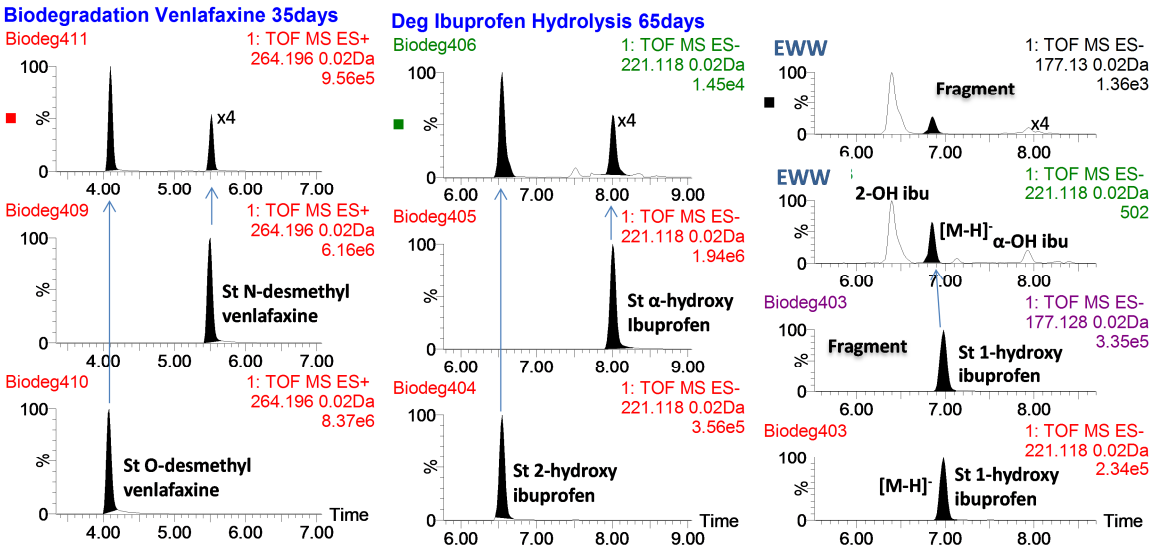

Figura 5.3 nw-XICs de VB1a (O-desmetil venlafaxina), VB1b (N-desmetil venlafaxina), IbSW2a (2-hidroxi ibuprofeno), IbSW2b (α-hidroxi ibuprofeno) y Ib1 (1-hidroxi ibuprofeno) en muestra degradadas (o efluente para Ib1) y en patrones de referencia.

6
CAPÍTULO

DESARROLLO DE METODOLOGÍA ANALÍTICA DE SCREENING BASADA EN LC-QTOF MS PARA LA INVESTIGACIÓN DE FÁRMACOS DE USO VETERINARIO Y HUMANO EN PIENSOS

6.1 Introducción

6.2 Artículo Científico 7: *Qualitative screening of 116 veterinary drugs in feed by liquid chromatography–high resolution mass spectrometry: potential application to quantitative analysis*. Food Chemistry, (2014) 160 313-320

6.3 Discusión de los resultados

6.1 Introducción

Los fármacos se usan en los animales, al igual que en las personas, para tratar y controlar enfermedades. Sin duda, proteger la salud de los animales ayuda a proteger la salud humana. Aproximadamente el 60% de las enfermedades que afectan a los humanos provienen de los animales, de manera que existe una fuerte relación entre la salud animal y la humana. Según organizaciones de salud de todo el mundo, entre las que se encuentran la Organización Mundial de la Salud (*World Health Organization*) y los Centros para el Control de Enfermedades de los Estados Unidos (*Centers for Disease Control*), mantener la salud en ambas poblaciones es crucial (Huet, 2013).

Los hábitos de consumo de alimentos han sufrido importantes cambios a lo largo de los años. Por consiguiente, es imprescindible un control eficaz de la producción, a fin de evitar las consecuencias perjudiciales que derivan de las enfermedades y los daños a la salud pública provocados por el consumo de alimentos contaminados, que contengan aditivos o residuos de productos prohibidos, que además podrían poner en riesgo la economía de un sector o de un país.

Un claro ejemplo sobre las consecuencias derivadas de la presencia de productos prohibidos está relacionado con el clembuterol detectado en productos cárnicos. Este β-agonista fue uno de los fármacos ilegales más usados en animales productores de alimentos en los años 90. Los β-agonistas, entre los que también se encuentran el clempenterol, la ractopamina, el brombuterol, el mabuterol o el mapenterol (**Figura 6.1**), provocan respuestas específicas en una variedad de tejidos mediante la unión con alta afinidad y alta especificidad a receptores β-adrenérgicos. La utilización de β-agonistas en alimentación animal presenta una serie de ventajas relacionadas no sólo con la mejora de la productividad y crecimiento del animal, sino también con la calidad de la carne, consiguiéndose mayores tejidos magros. Sin embargo, los residuos de agentes β-agonistas en tejidos animales (preferentemente hígado) destinados a consumo, constituyen un riesgo potencial para la salud humana. La carne procedente de animales tratados con clembuterol puede causar intoxicación e incluso la muerte de los consumidores. Los efectos tóxicos agudos son claros y predecibles teniendo en cuenta el modo de acción de los compuestos β-agonistas.

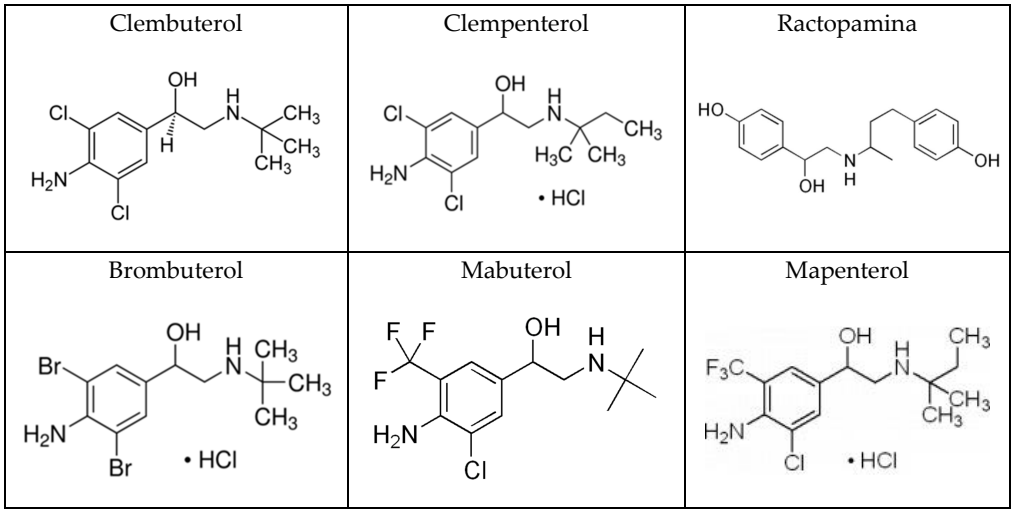

Figura 6.1 Estructuras químicas de fármacos veterinarios β-agonistas.

El clembuterol ha estado vinculado a varios brotes de enfermedades relacionadas con los alimentos de origen animal en Europa:

- 1990: En España 135 personas manifestaron síntomas tras consumir hígado de vacuno conteniendo residuos de clembuterol (0.16-0.30 ppm) procedentes de animales tratados de forma fraudulenta.
- 1991: En Francia 22 personas resultaron intoxicadas por consumir hígado de ternera con residuos de clembuterol.
- 1994: En España, de nuevo, el hígado de ternera estuvo implicado en la intoxicación de 127 personas.
- 1995: En Italia se encontró clembuterol (> 0.5 ppm) en filetes de buey y en bistecs del cuarto trasero, causando la intoxicación de 16 personas.
- 1996: En Italia 62 personas fueron hospitalizadas en unidades de urgencia por el consumo de carne de vacuno conteniendo clembuterol.

El caso del clembuterol es sólo un ejemplo que muestra la importancia de controlar potenciales contaminantes/productos prohibidos en alimentos y piensos.

Como es bien sabido, éste y otros muchos medicamentos seguirán estando disponibles, legal o ilegalmente, para prevenir, controlar y tratar enfermedades en animales destinados al consumo. Por ello, es fundamental que se desarrollen metodologías analíticas avanzadas capaces de detectar e identificar el mayor número posible de compuestos no autorizados y potencialmente peligrosos, ayudando a proteger de ese modo, tanto la salud animal como la salud humana.

El trabajo que se presenta a continuación, *Artículo Científico 7*, surgió a raíz de una colaboración con el Laboratorio de Salud Pública de Valencia. En este trabajo se muestra el desarrollo, validación (cualitativa) y aplicación de un método rápido,

sensible y selectivo para el *screening* de 116 fármacos humanos y veterinarios en pienso animal. El método está basado en una etapa de extracción del pienso, utilizando acetonitrilo acidificado, previa al análisis por UHPLC-QTOF MS. La metodología desarrollada se validó cualitativamente, estableciéndose dos parámetros fundamentales: el límite de detección (*Screening Detection Limit,* SDL) y el límite de identificación (*Limit Of Identification,* LOI). Además del análisis cualitativo, se llevó a cabo un estudio semi-cuantitativo de los compuestos detectados en las muestras de piensos analizadas (en total 22 muestras de piensos para ganado vacuno, porcino, avícola, caprino y cunícola).

6.2 Artículo Científico 7

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Analytical Methods

Qualitative screening of 116 veterinary drugs in feed by liquid chromatography–high resolution mass spectrometry: Potential application to quantitative analysis

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ABSTRACT

Veterinarian and human pharmaceuticals may be intentionally added to animal feed to enhance animal production. Monitoring these substances is necessary for protecting the consumers. In this work, a screening method covering 116 human and veterinary drugs has been developed and validated in five types of animal feed at 0.02 and 0.2 mg kg^{-1}. After a simple extraction and dilution, the samples were analysed by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF MS). Nearly all compounds tested were detected at 0.02 mg kg^{-1}, based on the presence of the accurate-mass (de)protonated molecule. However, the identification using a second accurate-mass ion was more problematic at this level. Finally, the procedure was applied to 22 feed samples, where trimethoprim, robenidine, or α- and β-nandrolone were detected and identified. The potential applicability of the method to quantitative analysis of the compounds detected in the samples was also evaluated.

1. Introduction

Over the last decades, livestock production has increased notably, mainly due to intensive farming. Veterinary drugs have been extensively used in animal husbandry, both for prophylactic and therapeutic purposes (Lopes et al., 2012). It is estimated that 6051 tons of various active substances are used as veterinarian medicines in the European Union to enhance animal production (Kools, Moltmann, & Knacker, 2008). Human pharmaceuticals (especially antibiotics) can also be added to animal feed, because of their commercial availability and low cost. In this context, pharmaceutical dosing must be carefully monitored to achieve a compromise between the agronomic results and the negative environmental and sanitary consequences of releasing these drugs to agro ecosystems (Granados-Chinchilla, Sánchez, García, & Rodríguez, 2012). The control of these substances is also necessary for protecting the consumers. Animal feeds must have the required quality and be appropriate from a nutritional point of view. They must be safe, i.e., free from contaminants and residues in general, and from residues of veterinary drugs in particular. The case of antimicrobials is of particular concern, as they might provoke allergies and contribute to the development of resistant bacterial strains if they reach the food chain (Borràs et al., 2011).

Some substances are banned, while others are authorised as long as their concentrations in food of animal origin remain below certain established limits. Thus, Directive 2002/32/EC regulated the measures on undesirable substances in animal feed (European Commission, 2002a). Regulation (EC) 1831/2003 banned the use of all antibiotics other than coccidiostatics and histomonostats as feed additives from 1 January 2006 (European Commission, 2003). Recently, Directive 2009/8/EC established maximum levels of unavoidable carry-over for these compounds in non-target feed (European Commission, 2009).

Drugs can reach feeds in three ways: authorised drugs (for therapeutic and prophylactic purposes), unauthorised drugs (as grow promoters to increase yield) and unintentional (as a result of the so-called cross-contamination) (Borrás et al., 2012). Although several different analytical methods based on liquid chromatography coupled to fluorescence or ultraviolet detection

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QUALITATIVE SCREENING OF 116 VETERINARY DRUGS IN FEED BY LIQUID CHROMATOGRAPHY–HIGH RESOLUTION MASS SPECTROMETRY: POTENTIAL APPLICATION TO QUANTITATIVE ANALYSIS

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Highlights

- Rapid screening was developed for human and veterinary drugs by UHPLC-QTOF MS in bovine, rabbit, poultry, goat and pork feeds.
- Qualitative validation was made for 116 drugs in different animal feeds at 0.02 and 0.2 mg kg^{-1}.
- Accurate-mass full-spectra data allowed a retrospective searching of additional compounds in the samples.
- Analysis of 22 commercial feeds showed several positive findings such as prohibited α- and β-nandrolone.

ABSTRACT

Veterinarian and human pharmaceuticals may be intentionally added to animal feed to enhance animal production. Monitoring these substances is necessary for protecting the consumers. In this work, a screening method covering 116 human and veterinary drugs has been developed and validated in five types of animal feed at 0.02 and 0.2 mg kg^{-1}. After a simple extraction and dilution, the samples were analysed by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QTOF MS). Nearly all compounds tested were detected at 0.02 mg kg^{-1}, based on the presence of the accurate-mass (de)protonated molecule. However, the identification using a second accurate-mass ion was more problematic at this level. Finally, the procedure was applied to 22 feed samples, where trimethoprim, robenidine, or α- and β-nandrolone were detected and identified. The potential applicability of the method to quantitative analysis of the compounds detected in the samples was also evaluated.

Keywords

Veterinary drugs, animal feed, liquid chromatography, time-of-flight mass spectrometry, screening, qualitative validation.

1. INTRODUCTION

Over the last decades, livestock production has increased notably, mainly due to intensive farming. Veterinary drugs have been extensively used in animal husbandry, both for prophylactic and therapeutic purposes (Lopes et al., 2012). It is estimated that 6051 tons of various active substances are used as veterinarian medicines in the European Union to enhance animal production (Kools, Moltmann, & Knacker, 2008). Human pharmaceuticals (especially antibiotics) can also be added to animal feed, because of their commercial availability and low cost. In this context, pharmaceutical dosing must be carefully monitored to achieve a compromise between the agronomic results and the negative environmental and sanitary consequences of releasing these drugs to agro ecosystems (Granados-Chinchilla, Sánchez, García, & Rodríguez, 2012). The control of these substances is also necessary for protecting the consumers. Animal feeds must have the required quality and be appropriate from a nutritional point of view. They must be safe, i.e., free from contaminants and residues in general, and from residues of veterinary drugs in particular. The case of antimicrobials is of particular concern, as they might provoke allergies and contribute to the development of resistant bacterial strains if they reach the food chain (Borràs et al., 2011).

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Drugs can reach feeds in three ways: authorised drugs (for therapeutic and prophylactic purposes), unauthorised drugs (as grow promoters to increase yield) and unintentional (as a result of the so-called cross-contamination) (Borrás et al., 2012). Although several

different analytical methods based on liquid chromatography coupled to fluorescence or ultraviolet detection have been developed, the most recent methodology relies on mass spectrometry (Capitan-Vallvey et al., 2007 and Kot-Wasik and Wasik, 2005) or, preferably, tandem mass spectrometry detection with triple quadrupole (Boscher et al., 2010, Cronly et al., 2010, De Baere and De Backer, 2007, Van Holthoon et al., 2010 and Vincent et al., 2008) or ion trap analysers (Kantiani et al., 2010 and Xu et al., 2011), because of the high selectivity and sensitivity provided by this technique. However, most of methods developed until now deal with a limited number of compounds, generally belonging to the same family. Among the most studied are the quinolones antimicrobials (Borrás et al., 2012, Boscher et al., 2010 and Xu et al., 2011), sulphonamides (Kantiani et al., 2010 and Lopes et al., 2012), macrolides (Boscher et al., 2010), β-lactams (Boscher et al., 2010, Kantiani et al., 2010 and Van Holthoon et al., 2010) or tetracyclines (Boscher et al., 2010 and Granados-Chinchilla et al., 2012). In these cases, specific sample treatments are normally applied.

The large number of available drugs has caused an increase in the number of analytes to be monitored. Under this situation, it is advisable to perform sample extractions as generic as possible in order to widen the scope of the method and to include as many analytes as possible. For large screening purposes, an alternative to MS/MS is the application of full scan techniques based on high resolution mass spectrometry (HRMS), using QTOF (Deng et al., 2011, Nácher-Mestre et al., 2013, van der Heeft et al., 2009 and Villar-Pulido et al., 2011) or Orbitrap (Kaufmann et al., 2011 and van der Heeft et al., 2009) analysers, which have opened new possibilities for analysis of many different organic contaminants/residues in matrices like milk (Freitas et al., 2013 and Stolker et al., 2008) urine (León et al., 2012), water (Díaz, Ibáñez, Sancho, & Hernández, 2013) or feed (Aguilera-Luiz et al., 2013, Martínez-Villalba et al., 2013 and Nácher-Mestre et al., 2013). As illustrative examples, Martínez-Villalba et al. (2013) and Nácher-Mestre et al. (2013) carried out a qualitative validation for chicken and fish feed, respectively, using HRMS. Aguilera-Luiz et al. (2013), made the validation for around 60 veterinary drugs and 150 pesticides in chicken, hen, rabbit and horse feed.

The objective of this work is to investigate the potential of UHPLC coupled to hybrid analyser QTOF MS for large screening (i.e. detection and identification of the compound detected) of human and veterinary drugs in different animal feeds (bovine, rabbit, poultry, goat and pork). This analyser allows to work under MS^E mode, which allows the simultaneous acquisition of accurate-mass full-spectrum acquisition data at low (LE) and high (HE) collision energy. MS^E mode provides useful information on the parent molecule (commonly the (de)protonated molecule, in the LE function, as this is the main ion observed in electrospray source under negative or positive ionisation mode), and on the main fragment ions (commonly in the HE function, where fragmentation is promoted) (Díaz et al., 2013 and Hernández et al., 2011). A subset of around 120 compounds from different chemical families has been selected for evaluation of the screening methodology. Once validated, the screening has been widened to search for around 530 additional pharmaceuticals, which reference standards were unavailable in our laboratory, and applied to 22 commercial feed samples to test its applicability. The possibilities to perform quantitative analysis for those positive pharmaceuticals found in animal feeds using the same instrument have been also evaluated.

2. EXPERIMENTAL

2.1. Reagents and chemicals

Reference compounds (Table S1) were purchased from Fort Dodge Veterinaria (Gerona, Spain), Vetoquinol Industrial (Madrid, Spain), Aventis Pharma (Madrid, Spain), Sigma Aldrich (St Louis, MO, USA), Cerilliant (Round Rock, TX, USA), Dr. Ehrenstorfer (Augsburg, Germany), Riedel-de Haën (Seelze, Germany), National Measurement Institute (Pymble, Australia), Witega (Berlin, Germany), and Fluka (Buchs, Switzerland). All reference materials had purities higher than 98% (w/w), except for marbofloxacin and pefloxacin, which had purities higher than 93%. Isotopically labelled internal standards (ILIS) fenilbutazone-d_{10}, robenidine-d_8, 4,4′dinitrocarbanilide-d_8 (DNC-d_8), amphetamine-d_6, benzoylecgonine-d_3 and carbamazepine epoxide-d_{10} were obtained from Cerilliant, and CDN Isotopes (Quebec, Canada).

HPLC-grade methanol (MeOH), acetonitrile (ACN) and sodium hydroxide (>99%) were purchased from ScharLab (Barcelona, Spain). Formic acid (HCOOH) (>98% w/w) was obtained from Fluka. Leucine enkephalin was purchased from Sigma Aldrich. HPLC-grade water was obtained from deionised water passed through a Milli-Q Gradient A10 (18.2 MΩ cm) water purification system (Millipore, Bedford, MA, USA).

2.2. Instrumentation

An Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) was interfaced to a QTOF mass spectrometer (QTOF Xevo G2, Waters Micromass, Manchester, UK) using an orthogonal Z-spray electrospray interface. The LC separation was performed using Acquity UPLC BEH C18 1.7 μm particle size analytical column of 100 × 2.1 mm (from Waters), at a flow rate of 0.3 mL min^{-1}. The mobile phases used were A H_2O and B MeOH, both with 0.01% (v/v) HCOOH. The percentage of MeOH was linearly increased as follows: 0 min, 10%; 14 min, 90%; 16 min, 90% and 16.01 min, 10%. The total run time was 18 min. The injection volume was 50 μL. Nitrogen (Praxair, Valencia, Spain) was used as both the drying gas and the nebulising gas. The desolvation gas flow rate was set at 1000 L h^{-1}. The resolution of the TOF mass spectrometer was ~20,000 at full

width half maximum (FWHM) at *m/z* 556. MS data were acquired over a *m/z* range of 50–1200 in a scan time of 0.3 s. Capillary voltages of 0.7 and −1.7 kV were used in positive and negative ionisation modes, respectively. A cone voltage of 25 V was applied. The collision gas was argon (99.995%, Praxair). The interface temperature was set to 650 °C and the source temperature to 130 °C. The column temperature was set to 40 °C and the samples to 5 °C. For MS^E experiments, two acquisition functions with different collision energies were created: the low-energy (LE) function with a collision energy of 4 eV, and the high energy (HE) function with a collision energy ramp ranging from 15 to 40 eV. The same cone voltage (25 V) and collision energy ramp was used for additional MS/MS experiments.

Calibration of the mass-axis from *m/z* 50 to 1200 was conducted daily with a 1:1 mixture of 0.05 M NaOH/5% (v/v) HCOOH diluted (1:25) with water/ACN (20:80 v/v).

For automated accurate mass measurement, the lock-spray probe was employed, using as lockmass leucine enkephalin (2 mg L^{-1}) in ACN/water (50/50) at 0.1% HCOOH, pumped at 20 µL min^{-1} through the lock-spray needle. The leucine enkephalin $[M+H]^+$ ion (*m/z* 556.2771) and its fragment ion (*m/z* 278.1141) for positive ionisation mode, and $[M–H]^-$ ion (*m/z* 554.2615) and its fragment ion (*m/z* 236.1035) for negative ionisation, were used for recalibrating the mass axis and to ensure a robust accurate mass measurement over time.

The data station operating software was MassLynx version 4.1 (Waters).

2.3. Feed samples

A total of 10 different feed samples were used for method validation (2 bovine, 2 rabbit, 2 poultry, 2 goat and 2 pork).

In a subsequent step, the developed procedure was applied to other feed samples to test its applicability. 22 feed samples (12 bovine, 3 rabbit, 2 poultry, 2 goat and 3 pork) were collected in polyethylene high-density bottles from farms located in Spanish

Mediterranean area (Valencia and Castellon provinces). Samples were stored at −18 °C. Before analysis, samples were thawed at room temperature and triturated with a crusher Super JS from Moulinex (Bagnolet Cedex, France).

2.4. Extraction procedure

Five grams of homogenised feed sample were accurately weighed (precision 0.1 mg) directly in centrifuge tubes (50 mL) and extracted with 10 mL of acetonitrile 1% HCOOH, using a vortex for 1 min. Then, the mixture was mechanically shaken end-over-end for 1 h. After that, samples were sonicated (15 min) and centrifuged at 4500 rpm for 10 min. A 2-mL aliquot of supernatant was transferred to an Eppendorf tube, and a second centrifugation was performed at 12000 rpm (12,074 *g*) for 10 min. Finally, the extract was ten-fold diluted with Milli-Q water (100 µL extract + 900 µL water) and injected in the system. No microfiltration was made to avoid potential losses of compounds in this step.

2.5. Qualitative validation protocol

In this work, method validation was performed following the strategy described in the literature (Díaz et al., 2013 and Nácher-Mestre et al., 2013).

Ten different animal feeds (five types of matrices, two feed samples for each matrix) were spiked with a mix solution of the test analytes in methanol at two levels, 0.02 and 0.2 mg kg^{-1}, let stand overnight, and analysed together with their non-spiked samples (blanks). The final concentrations in the ten-fold diluted extracts were 1 and 10 µg L^{-1}, respectively.

The screening detection limit (SDL) and the limit of identification (LOI) were investigated as the main validation parameters to estimate the threshold concentration at which detection and identification become reliable, respectively. The SDL was established as the lowest concentration level tested for which a compound was detected in all samples, using the most abundant ion (typically, the (de)protonated molecule) measured at its exact mass (mass error lower than ±2 mDa) and at the expected retention time (±2.5% RT deviation tolerance). The LOI was established as the lowest concentration tested for

which a compound was satisfactorily identified in all spiked samples. The identification criterion was the presence of, at least, two *m/z* ions in either the LE or HE function at the expected retention time measured at their exact mass.

The terms SDL and LOI would be equivalent to the definition of "screening target concentration" and "detection capability", respectively, provided by the CRL's 2010 guideline (CRLs, 2010).

3. RESULTS AND DISCUSSION

3.1. Database building

A large number of pharmaceuticals, which could be potentially present in animal feed, were selected as target compounds, with *m/z* values ranging from 137.0239 ($[M-H]^-$ ion of salicylic acid) to 934.5739 ($[M+H]^+$ion of maduramicine). For those pharmaceuticals which reference standard was available in our lab (116 compounds), empirical data was obtained after injection into the UHPLC-QTOF MS, following the working conditions reported in Díaz, Ibáñez, Sancho, and Hernández (2011) (Díaz et al., 2011). These compounds were afterwards used in the validation experiments. Table S1 shows the compound name, ionisation mode, retention time (min) and exact mass for (de)protonated compounds, and the elemental compositions for their main fragment ions. 93 out of 116 analytes were detected in positive ionisation mode, whereas 9 were in negative mode; 14 compounds were detected in both modes. For the remaining compounds, which standards were unavailable (530 approx), information on elemental composition was included in the database, for future screening in real samples. The database included anabolic substances (such as thyrostatic compounds, stilbenes, stilbenes derivatives, 17-β-estradiol and ester derivatives) as well as β-agonists, prohibited according to (European Commission, 2008). Compounds with maximum levels established (coccidiostatics and histomonostats) (European Commission, 2002a) and other veterinarian and human pharmaceuticals not regulated, mainly antimicrobials, were also included.

3.2. Qualitative validation results

Five different feed matrices were tested in method validation: bovine, rabbit, poultry, goat and pork. Two samples of each type were spiked with the mix of 116 pharmaceuticals at two concentration levels (0.02 and 0.2 mg kg^{-1}). These levels were selected accordingly with the feed regulation (Bruni & Ferreira, 2008). Table S2 shows the SDL and LOI obtained for each analyte in each matrix. A summary of this table is also shown in Fig. S1. As it can be seen, around 75% of compounds could be detected at

0.2 mg kg^{-1} (see accumulated,Fig. S1) while the percentage of detection decreased down to 40% at 0.02 mg kg^{-1}. It is noteworthy the great differences observed between the matrices studied. As shown in Fig. S2, significant differences in the signal response (e.g. in sensitivity) were found between standards in solvent and in the five feed samples (all at a concentration of 0.2 mg kg^{-1}), as illustrated for six of the compounds investigated. This made that, although around 90% of the compounds were detected in each matrix individually at 0.2 mg kg^{-1}, only 75% could be fully validated at this level, i.e. only 75% were detected in all matrices. The highest number of compounds detected corresponded to poultry and pork feeds (around 70 pharmaceuticals). On the contrary, bovine, rabbit and goat feeds appeared as the most problematic matrices, which may be considered more complex than others, likely due to their different fatty content (Aguilera-Luiz et al., 2013). Nine pharmaceuticals (diethylstilbestrol, 16-β-hydroxystanozolol, amoxicillin, cefaclor, cefadroxil, cephalexin, abamectine, doramectine and omeprazole) were not detected in any of the spiked samples. These compounds might not be stable under the extraction conditions assayed or might be highly affected by matrix effects.

Overall, the reliable identification using two accurate-mass ions was feasible for 55% of compounds at 0.2 mg kg^{-1}. This value drastically decreased down to 10% at 0.02 mg kg^{-1}, showing the great difficulties to obtain a second ion at low analyte concentration in this type of matrices. Regarding poultry and pork feeds around 40% of compounds could be identified at the lower level; yielding more than 70% of the pharmaceuticals identified at 0.2 mg kg^{-1}. The apparently more complex goat, rabbit and bovine feed matrices allowed the identification of around 60% of the compounds analysed at the 0.2 mg kg^{-1} level. Concentrations higher than 0.2 mg kg^{-1} were not tested. Surely, most of compounds which were detected but could not be identified at 0.2 mg kg^{-1} because of the low sensitivity for its fragment ion might be identified at higher concentration levels.

In this work, typically the (de)protonated molecule and at least one collision induced dissociation (CID) fragment ion, in either, the LE or HE functions, were used for detection and identification, respectively. However, several compounds presented poor or none fragmentation as result of the compromise collision energy ramp applied. This was the

case of ivermectin or ipromidazole, amongst others. In those cases, when fragment ions were not observed, only SDL could be set-up (12 out of 116 compounds).

Table 1 shows the total SDL for the 10 samples under study (i.e. SDL means detected in all 10 samples analysed), and illustrates the applicability of the screening for detection of veterinary drugs.

Table 1. Screening detection limit (SDL) and limit of identification (LOI) (both in mg kg^{-1}) for studied compounds.

Stilbenes	**SDL**	**LOI**	**Nitromidazoles**	**SDL**	**LOI**	**Amphenicols (cont.)**	**SDL**	**LOI**
Dienestrol	>0.2	>0.2	Chloramphenicol	0.02	0.2	Florfenicol	0.2	>0.2
Hexestrol	>0.2	>0.2	DMZ	0.02	>0.2	Florfenicol amine	>0.2	>0.2
Steroids	**SDL**	**LOI**	Furaltadone	0.2	>0.2	Flumequine	0.02	0.2
Boldione	>0.2	>0.2	HMMNI	>0.2	>0.2	Lincomycin	0.02	0.2
α-Boldenone	0.02	0.2	IPZOH	0.02	0.02	Nalidixic acid	0.02	0.2
α-Nandrolone	0.02	>0.2	MNZOH	>0.2	>0.2	Norfloxacin	0.2	0.2
AED	>0.2	>0.2	IPZ	0.2	>0.2	Ofloxacin	0.2	0.2
β-Boldenone	0.2	>0.2	MNZ	>0.2	>0.2	Oxacillin	0.2	>0.2
β-Nandrolone	0.2	0.2	RNZ	>0.2	>0.2	Oxolinic acid	0.2	0.2
Stanozolol	0.02	>0.2	**Amphenicols**	**SDL**	**LOI**	Oxytetracycline	>0.2	>0.2
RALs	**SDL**	**LOI**	Ampicillin	0.2	>0.2	Pefloxacin	0.02	0.2
α-Zeranol	>0.2	>0.2	Cefotaxim	0.2	0.2	Penicillin G	0.2	0.2
β-Zeranol	0.2	>0.2	Ceftriaxone	>0.2	>0.2	Pipedimic acid	0.2	0.2
ZAN	>0.2	>0.2	Cefuroxime	>0.2	>0.2	Piperacillin	0.2	0.2
β-Agonists	**SDL**	**LOI**	Chlortetracycline	>0.2	>0.2	Roxythromycin	0.02	0.2
Brombuterol	0.02	0.2	Ciprofloxacin	0.2	0.2	Sarafloxacin	0.2	>0.2
Clenbuterol	0.02	0.2	Clarithromycin	0.02	0.02	Sulfadoxine	0.02	0.2
Clenpenterol	0.02	0.2	Cloxacillin	0.2	0.2	Sulfamethoxazole	0.02	0.2
OH clenbuterol	0.02	0.02	Dicloxacillin	0.2	>0.2	Tetracycline	>0.2	>0.2
Mabuterol	0.02	0.02	Doxycycline	>0.2	>0.2	Thiamphenicol	0.2	>0.2
Mapenterol	0.02	0.02	Enrofloxacin	0.2	0.2	Trimethoprim	0.02	0.02
Ractopamine	0.02	0.2	Erythromycin A	0.2	>0.2			

Table 1 (Cont.). Screening detection limit (SDL) and limit of identification (LOI) (both in mg kg^{-1}) for studied compounds.

Avermectins	**SDL**	**LOI**
Emamectin B1a	0.2	0.2
Eprinomectin	0.2	>0.2
Ivermectin	>0.2	>0.2
Levamisole	0.02	0.2
Moxidectin	>0.2	>0.2
Coccidiostats	**SDL**	**LOI**
Maduramicine	0.02	0.2
Monensin	0.2	0.2
Narasin	0.02	>0.2
Robenidine	0.02	0.2
Salinomycin	0.2	>0.2
Sedatives	**SDL**	**LOI**
Acepromazine	0.02	0.2
Alprazolam	0.02	0.2
Azaperol	0.02	>0.2
Azaperone	0.02	0.2
Carazolol	0.02	0.02
Chlorpromazine	0.02	0.02
Combelen	0.02	0.2

NSAIDs	**SDL**	**LOI**
4-acetylamino-antipyrine	0.02	0.2
4-amino-antipyrine	>0.2	>0.2
4-formylamino-antipyrine	0.2	0.2
Diclofenac	0.2	0.2
Ibuprofen	>0.2	>0.2
Ketoprofen	0.02	0.2
Mefenamic acid	0.02	0.2
Naproxen	0.2	>0.2
Oxyphenylbutazone	0.2	>0.2
Phenylbutazone	>0.2	>0.2
Salicylic acid	0.02	>0.2
Corticoids	**SDL**	**LOI**
Betamethasone/Dexamethasone	0.2	>0.2
Flumethasone	0.2	>0.2
Methylprednisolone	>0.2	>0.2
Parasiticide	**SDL**	**LOI**
Leucomalachite green	>0.2	>0.2
Malachite green	0.2	0.2

Other pharmaceutical	**SDL**	**LOI**
Acetaminophen	>0.2	>0.2
Atorvastatin	0.2	>0.2
Bezafibrate	0.2	>0.2
Carbamazepine	0.02	>0.2
Enalapril	0.02	0.2
Gemfibrozil	0.2	0.2
Irbesartan	0.02	0.2
Lorazepam	0.2	0.2
Olanzapine	0.2	0.2
Pantoprazole	>0.2	>0.2
Pravastatin	0.2	0.2
Valsartan	0.2	0.2
Venlafaxine	0.02	0.02

3.3. Application to routine samples

A total of twenty-two feed samples (12 bovine, 3 rabbit, 2 poultry, 2 goat and 3 pork) were analysed following the developed procedure in order to evaluate its applicability. Up to 11 compounds were detected and properly identified. Table 2 shows a summary of the results obtained.

Quantification was only made for those compounds and matrices previously tested, and proven to have satisfactory recovery (between 60% and 120%) and RSD (<15%).

The most detected compounds were the salicylic acid, active metabolite of anti-inflammatory acetylsalicylic acid (aspirin), and the antimicrobial trimethoprim, which were found in 50% of the samples analysed. As an illustrative example, Fig. S3 shows the HE spectra of a reference standard of trimethoprim and a positive poultry feed sample. As it can be seen, both spectra perfectly matched, sharing up to 8 main fragment ions with mass errors lower than 2 mDa, at the expected retention time (3.6 min). Fig. 1 shows another example, the detection and identification of the antibiotic lincomycin in a pork feed sample. In this case, the ions corresponding to the protonated molecule and to its unique fragment ion (at m/z 407.2216 and m/z 126.1283, respectively) at the expected retention time were observed. In addition to the presence of the two ions with acceptable mass errors, the intensity ratio between the most abundant ion (i.e. the protonated molecule) and the fragment ion used for confirmation was also calculated obtaining a deviation of 1.3% in relation to the reference standard. This deviation was by far lower than the ±20% allowed by the EU guidelines for ion ratios between 1 and 2, giving even more reliability to the confirmation process (European Commission, 2002b).

Table 2. Positives found in 22 feed samples analysed by UHPLC-QTOF MS.

Compound	Positive findings									
	Bovine (n=12)		Rabbit (n=3)		Poultry (n=2)		Goat (n=2)		Pork (n=3)	
	Number of pos. findings	Conc (mg kg^{-1})	Number of pos. findings	Conc (mg kg^{-1})	Number of pos. findings	Conc (mg kg^{-1})	Number of pos. findings	Conc (mg kg^{-1})	Number of pos. findings	Conc (mg kg^{-1})
α-Nandrolone	2		-		2		1		2	
β-Nandrolone	1	0.3	-		-		-		-	
Robenidine	1	<0.2	2	40.4,31.2	-		-		-	
Chlortetracycline	1		-		1		-		-	
Oxytetracycline	-		2		-		-		1	52.4
Tetracycline	1		-		1		-		1	1.7
Doxicycline	-		1		-		-		1	18.7
Lincomycin	1	22.2	2		-		1		3	
Trimethoprim	5		2		1		1	1.2	2	
Florfenicol	2	0.5,0.4	-		-		-		-	
Salicylic acid	3	1.0	3		1		2		2	

Quantification was only made for those compounds and matrices previously tested, and proven to have satisfactory recovery (between 60 and 120%) and RSD (< 15%).

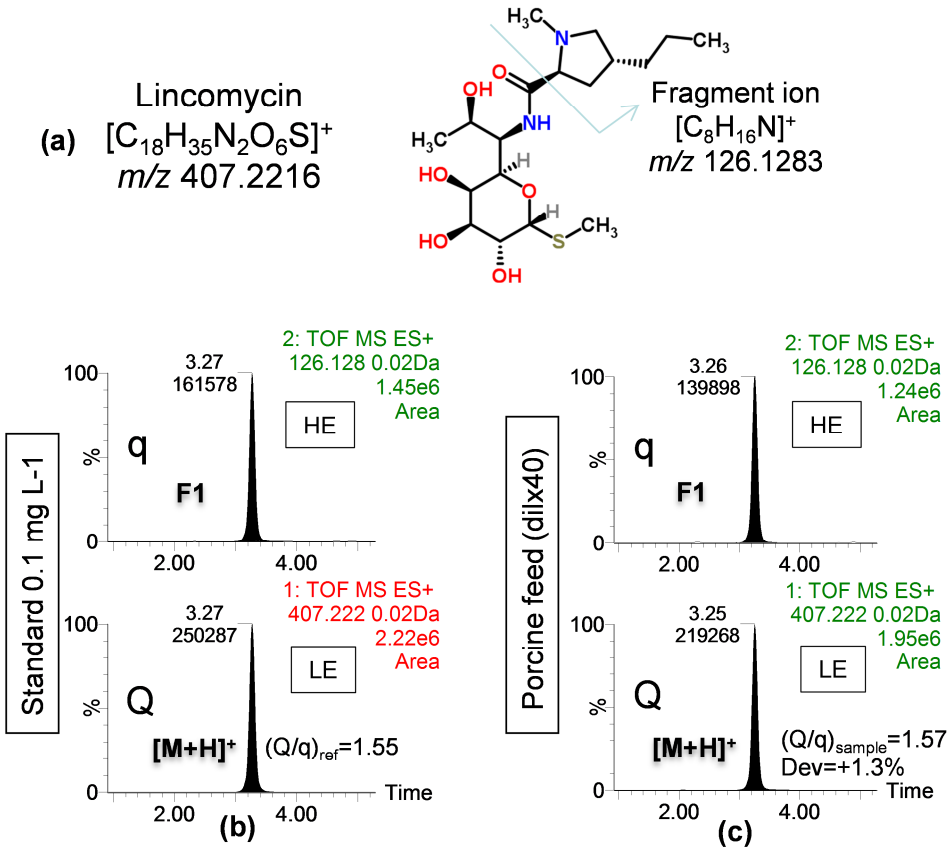

Fig. 1. (a) Lincomycin and its fragment ion structure. nw-XICs corresponding to the protonated molecule at LE and to its fragment ion at HE for (b) 0.1 mg L^{-1} reference standard and (c) positive pork feed sample.

Up to 4 tetracyclines (chlortetracycline, oxytetracycline, tetracycline and doxycycline) were found in several matrices, the highest levels being found in one of the pork feeds particularly oxytetracycline (Table 2).

The antibiotic florfenicol was also identified in 2 bovine samples. This antimicrobial is currently indicated for the treatment of bovine respiratory diseases (European Medicines Agency, 2009).

The detection of robenidine, a coccidiostat used for the control of protozoal infection, and regulated in the European Commission Directive EC 2009/8 (European Commission, 2009) was also of interest. This compound was detected in one bovine and two rabbit

feed samples. Fig. 2 illustrates the identification and quantification of this pharmaceutical in rabbit feed. As it can be seen, not only the protonated molecule but also two fragment ions were observed at the expected retention time. Robenidine-d_8 was used as isotope-labelled internal standard (ILIS) to compensate for matrix affects as well as possible variations in the instrument measurement. The concentration calculated for this sample was 40.4 mg kg^{-1} (see next section for more details). Although this value by far exceeds the maximum content of 0.7 mg kg^{-1} established by the Directive (European Commission, 2010), a subsequent Commission Regulation (Commission implementing Regulation (EU) No. 532/2011 of 31 May 2011 concerning the authorisation of robenidine hydrochloride as a feed additive for rabbits for breeding and rabbits for fattening (holder of authorisation Alpharma Belgium BVBA) and amending Regulations (EC) No. 2430/1999 and (EC) No. 1800/2004) concerning the authorisation of robenidine hydrochloride as a feed additive for rabbits for breeding and rabbits for fattening, establishes the maximum content in 66 mg of active substance kg^{-1} of complete feeding stuff, until June 2021 (European Commission, 2011).

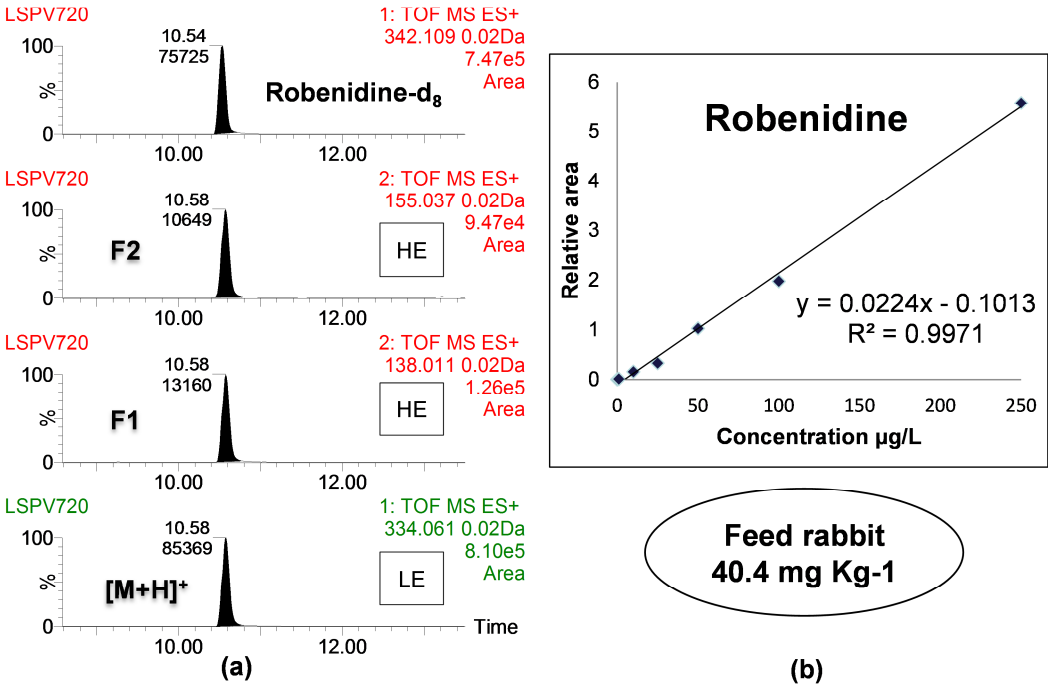

Fig. 2. Positive finding of the coccidiostatic robenidine in a rabbit feed. (a) nw-XICs at 20 mDa mass window for the protonated molecule at LE and its two main fragment ions at HE. (b) Calibration curve obtained using relative responses, with robenidine-d_8 as internal standard.

The most remarkable was the detection of the steroids α-nandrolone and β-nandrolone, found in 7 and 1 out of 22 feed samples analysed, respectively (Table 2). These compounds were banned by Directive 2008/97/EC European Parliament and of the Council of 19 November 2008 amending Council Directive 96/22/EC concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyrostatic action and of beta-agonists (European Commission, 2008). The identification of the hormone α-nandrolone in bovine feed is shown in Fig. 3, where the protonated molecule and two fragment ions were detected in the LE function. To support the confirmation of its identity, the intensity ratio was calculated too, obtaining a deviation of –44% for the main fragment ion (F1) which is lower than ±50% allowed by the EU guidelines for ion ratios ⩾10 (European Commission, 2002b).

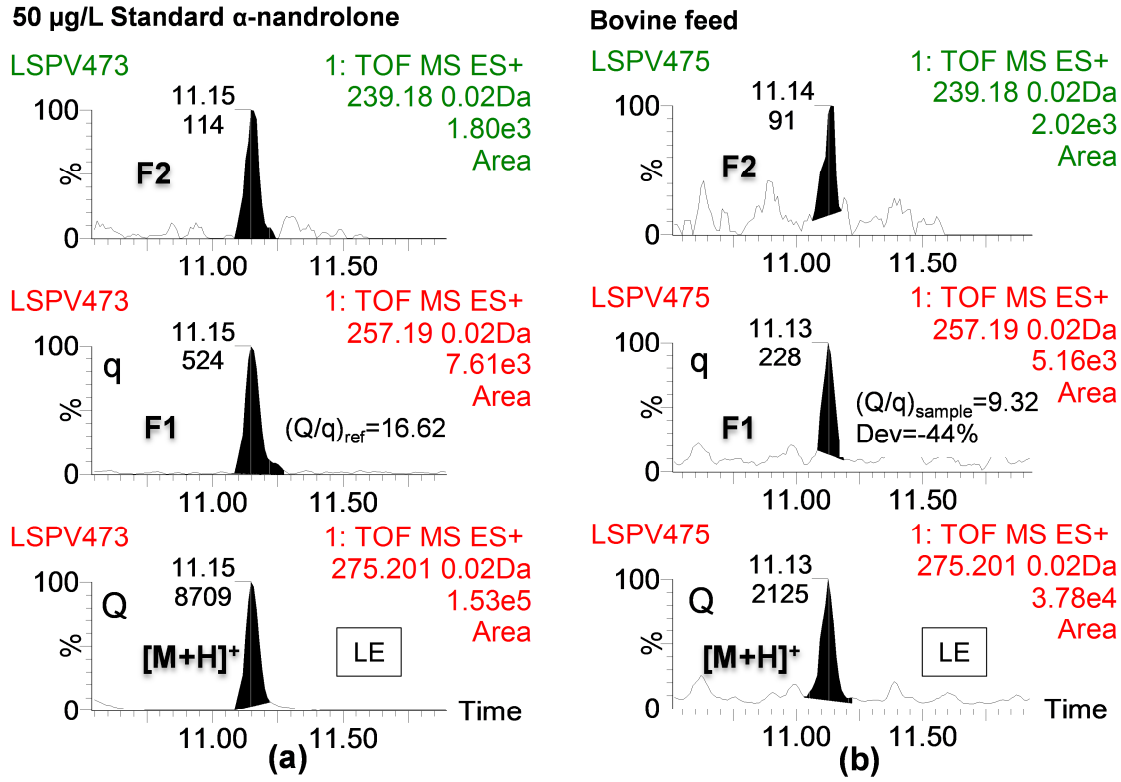

Fig. 3. Positive finding of α-nandrolone in a bovine feed. nw-XICs at 20 mDa mass window for the protonated molecule and two fragment ions at LE for (a) reference standard and (b) bovine feed.

Although the LOI could not be established to be 0.2 mg kg^{-1} for some compounds (i.e., the compound could not be identified at this level in spiked samples), they were however identified in the feed samples analysed. This means that their concentration in the samples was above the levels tested for LOI (0.02 and 0.2 mg kg^{-1}). This was the case of chlortetracycline in poultry feed, doxycycline in rabbit and pork feed, tetracycline in poultry and bovine feed, and salicylic acid in poultry, bovine, rabbit and pork feed. With the data available from QTOF MS analysis, the identification of these compounds in the samples was certainly highly reliable.

In case of detecting a compound whose reference standard was not available in the laboratory, its tentative identification would be based on the ions observed (protonated molecule and fragment ions), their compatibility with the chemical structure of the candidate, and on the comparison with those ions reported in the literature (Díaz et al., 2013 and Díaz et al., 2012). In the present work, for all compounds detected in feed the reference standards were all available in our laboratory.

3.4. Evaluation of the applicability to quantitative analysis

Finally, an evaluation of the applicability of the screening to quantitative analysis was made for the compounds found in the feed samples: β-nandrolone, robenidine, chlortetracycline, tetracycline, oxytetracycline, doxycycline, lincomycin, trimethoprim, florfenicol and salicylic acid. This investigation was not possible for α-nandrolone due to the lack of reference standard at sufficiently high concentration (a mixture of several standards, at around 10 mg L^{-1}, was only available for this compound as a gift from the Laboratory of Public Health of Valencia).

For this purpose, bovine, poultry and pork feeds were spiked by triplicate at 0.2 and 2 mg kg^{-1}. Those feeds previously analysed and proven to not contain the analytes were selected as "blanks" to perform recovery experiments (one feed for each matrix by triplicate). In addition to spiked samples, "blank" samples, spiked only with an ILIS mix, were also processed to subtract the responses of possible positive compounds. Quantification was performed using calibration standards in solvent and relative

responses to ILIS for matrix effects correction. Unfortunately, robenidine was the only analyte among all tested for which its labelled compound (robenidine-d_8) was available at our laboratory. After several preliminary experiments, for the rest of compounds we selected other available ILIS that seemed to roughly compensate the matrix effect observed (Table S3).

The linearity of the method was studied in the range 1–250 μg L^{-1} for all selected compounds obtaining satisfactory correlation coefficients (greater than 0.99).

Regarding robenidine, satisfactory recoveries (61–93%) and precision (1–6%) were obtained at 0.2 mg kg^{-1} spiked level for the three matrices, and also at 2 mg kg^{-1} (recovery 90–98%, precision 3–6%) (Table S3). Nevertheless, for the other compounds, recoveries were not completely satisfactory in all matrices although they mostly varied between 60% and 120%. Tetracyclines could only be validated in pork feed at the highest level assayed. With few exceptions, RSD were satisfactory (below 15%) except for chlortetracycline in pork, and salicylic acid in bovine and poultry. It seemed that the analyte-labelled ILIS was required to ensure appropriate correction due to the strong matrix effect resulting from the matrix complexity and little sample manipulation. Fig. S2 shows the responses for six pharmaceuticals in solvent and in five feed matrices spiked at 0.2 mg kg^{-1}, illustrating the large signal differences observed between each feed matrix. Matrix effects correction using ILIS other than the labelled analyte was not always assured. Based on the experiments performed, we quantified the compounds detected in the samples only for those matrices previously tested, which recoveries ranged between 60% and 120% with RSD < 15% (see Table 2). Although rabbit feed was not tested in recovery experiments, a high robenidine concentration of 40.4 mg kg^{-1} could be reported due to the satisfactory recovery obtained in other matrices and to the availability of analyte ILIS.

The objective of the present work was to investigate the potential of LC-QTOF MS for screening of veterinary drugs. In the case that this methodology was finally used by reference laboratories and for official purposes, the appropriate guidelines should be taken into account, as for example the one established for community reference

laboratories residues (CRLs, 2010), or other guidelines applied in related fields (European Commission, 2013).

4. CONCLUSIONS

In this article, a multiclass, wide-scope, and rapid screening based on UHPLC-QTOF MS has been developed for human and veterinary pharmaceuticals in five types of animal feeds (bovine, rabbit, poultry, goat and pork). After extraction of the sample with acidified acetonitrile and direct analysis by QTOF MS, the wide majority of the 116 compounds tested were detected and correctly identified in all feed samples spiked at 0.2 mg kg^{-1}. Detection, based on the presence of the (de)protonated molecule, was also feasible in most cases at the lowest level tested (0.02 mg kg^{-1}), although identification using a second accurate-mass fragment ion, was problematic at this concentration due to the complexity of the sample matrices.

The screening procedure was applied to 22 feed samples, with the result of detecting and correctly identifying several antibiotics, such as florfenicol, robenidine or lincomycin; one of them, robenidine, included in the current legislation. Moreover, two hormones, α- and β-nandrolone, banned by the directive (European Commission, 2008), were also found in some feeds. Trimethoprim and salicylic acid, were the compounds more detected (50%) followed by lincomycin and α-nandrolone (32%).

The strong potential of LC-QTOF MS for wide-scope screening of veterinary drugs in feed has been proven in this work. Although the main applications of QTOF MS are directed towards detection and identification of the compounds, this technique might be also used for quantitative purposes. To explore this possibility, a preliminary work has been made with the compounds that were detected in the previous screening obtaining promising results. The availability of robenidine-d_8 used as ILIS allowed the reliable quantification of robenidine in the samples analysed, thanks to the satisfactory matrix effects correction, as demonstrated by the satisfactory recoveries and precision obtained at 0.2 and 2 mg kg^{-1} spiked levels. This compound was found in two rabbit feeds with a maximum concentration of 40.4 mg kg^{-1}. In the light of the results obtained in this work, a quantitative analysis might be also feasible using LC-QTOF MS, although more work is required to fully explore this possibility. This technique, similarly to the most widely used

for quantitative analysis in this field (i.e. LC–MS/MS), is notably affected by matrix effects, and therefore the use of ILIS greatly facilitates an accurate quantification.

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REFERENCES

M.M. Aguilera-Luiz, R. Romero-González, P. Plaza-Bolaños, J.L. Martínez Vidal, A. Garrido Frenich Wide-scope analysis of veterinary drug and pesticide residues in animal feed by liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry Analytical and Bioanalytical Chemistry (2013), pp. 1–11.

S. Borrás, J. Ríos-Kristjánsson, R. Companyõ, M. Prat Analysis of fluoroquinolones in animal feeds by liquid chromatography with fluorescence detection Journal of Separation Science, 35 (16) (2012), pp. 2048–2053.

S. Borràs, R. Companyó, M. Granados, J. Guiteras, A.M. Pérez-Vendrell, J. Brufau *et al.* Analysis of antimicrobial agents in animal feed Trends in Analytical Chemistry, 30 (7) (2011), pp. 1042–1064.

A. Boscher, C. Guignard, T. Pellet, L. Hoffmann, T. Bohn Development of a multi-class method for the quantification of veterinary drug residues in feedingstuffs by liquid chromatography–tandem mass spectrometry Journal of Chromatography A, 1217 (41) (2010), pp. 6394–6404.

A.T. Bruni, M.M.C. Ferreira Theoretical study of omeprazole behavior: Racemization barrier and decomposition reaction International Journal of Quantum Chemistry, 108 (6) (2008), pp. 1097–1106.

L.F. Capitan-Vallvey, A. Ariza, R. Checa, N. Navas Liquid chromatography–mass spectrometry determination of six 5-nitroimidazoles in animal feedstuff Chromatographia, 65 (5–6) (2007), pp. 283–290.

Community Reference Laboratories Residues (CRLs) Guidelines of 20th January 2010 for the validation of screening methods for residues of veterinary medicines.

M. Cronly, P. Behan, B. Foley, E. Malone, S. Earley, M. Gallagher *et al.* Development and validation of a rapid multi-class method for the confirmation of fourteen prohibited medicinal additives in pig and poultry compound feed by liquid chromatography–tandem mass spectrometry Journal of Pharmaceutical and Biomedical Analysis, 53 (4) (2010), pp. 929–938.

S. De Baere, P. De Backer Quantitative determination of amoxicillin in animal feed using liquid chromatography with tandem mass spectrometric detection Analytica Chimica Acta, 586 (1–2 SPEC. ISS.) (2007), pp. 319–325.

X. Deng, H. Yang, J. Li, Y. Song, D. Guo, Y. Luo *et al.* Multiclass residues screening of 105 veterinary drugs in meat, milk, and egg using ultra high performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry Journal of Liquid Chromatography and Related Technologies, 34 (19) (2011), pp. 2286–2303.

R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández Building an empirical mass spectra library for screening of organic pollutants by ultra-high-pressure liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry Rapid Communications in Mass Spectrometry, 25 (2) (2011), pp. 355–369.

R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández Target and non-target screening strategies for organic contaminants, residues and illicit substances in food, environmental and human biological samples by UHPLC-QTOF-MS Analytical Methods, 4 (1) (2012), pp. 196–209.

R. Díaz, M. Ibáñez, J.V. Sancho, F. Hernández Qualitative validation of a liquid chromatography-quadrupole-time of flight mass spectrometry screening method for organic pollutants in waters Journal of Chromatography A, 1276 (2013), pp. 47–57.

European Commission. (2002a). Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed, L 140/10. (consolidated version: 2002 L0032—ES—01.01.2012—014.002—1). Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:140:0010:0021: ES:PDF. (Accessed 25 Apr 2013).

European Commission European Union Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results Official Journal of the European Communities, L221 (2002), pp. 8–36 Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L: 2002:221:0008:0036:EN:PDF. (Accessed 19 February 2014).

European Commission Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition Official Journal of the European Union L, 268 (2003), p. 29 Available from: http://irmm.jrc.ec.europa.eu/SiteCollection Documents/EC-1831-2003.pdf. (Accessed 25 April 2013).

European Commission. (2008). Directive 2008/97/EC of the European Parliament and of the Council of 19 November 2008 amending Council Directive 96/22/EC concerning the prohibition on the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists. Available from: http://eur-lex.europa.eu/LexUriServ/ LexUriServ.do?uri=OJ:L:2008:318:0009:0011:EN:PDF. (Accessed 1 August 2013).

European Commission Commission Directive 2009/8/EC of 10 February 2009 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels of unavoidable carry-over of coccidiostats or histomonostats in nontarget feed Official Journal of the European Union L, 40 (2009), p. 19 Available from:

http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009: 040: 0019:0025:EN:PDF. (Accessed 25 Apr 2013).

European Commission, 2010. Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:015:0001:0072:ES: PDF. (Accessed 25 April 2013).

European Commission Commission implementing regulation (EU) 532/2011 of 31 May 2011 concerning the authorisation of robenidine hydrochloride as a feed additive for rabbits for breeding and rabbits for fattening (holder of authorisation Alpharma Belgium BVBA) and amending Regulations (EC) No 2430/1999 and (EC) No. 1800/2004 Official Journal of the European Union L, 146 (2011), p. 7 Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:146:0007:0010:EN:PDF. (Accessed 25 April 2013).

European Commission, 2013. SANCO/12571/2013 Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed. European Commission, Directorate General Health and Consumer Protection, 1 January 2014http://www.eurl-pesticides.eu/library/docs/allcrl/AqcGuidance_Sanco_2013_12571.pdf. (Accessed 19/02/2014).

European Medicines Agency. Committee for veterinary medicinal products: Florfenicol. http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500014274.pdf.

S.K.B. Freitas, A.P.S. Paim, P.T. de Souza e Silva Development of a LC-IT-TOF MS procedure to quantify veterinary drug residues in milk employing a QuEChERS approach Food Analytical Methods (2013), pp. 1–8.

F. Granados-Chinchilla, J. Sánchez, F. García, C. Rodríguez A novel green chemistry method for nonaqueous extraction and high-performance liquid chromatography detection of first-, second-, and third-generation tetracyclines, 4-epitetracycline, and tylosin in animal feeds Journal of Agricultural and Food Chemistry, 60 (29) (2012), pp. 7121–7128.

F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry Analytica Chimica Acta, 684 (1–2) (2011), pp. 87–97.

L. Kantiani, M. Farré, J.M.G.I. Freixiedas, D. Barceló Development and validation of a pressurised liquid extraction liquid chromatography-electrospray-tandem mass

spectrometry method for β-lactams and sulfonamides in animal feed Journal of Chromatography A, 1217 (26) (2010), pp. 4247–4254.

A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer Development of an improved high resolution mass spectrometry based multi-residue method for veterinary drugs in various food matrices Analytica Chimica Acta, 700 (1–2) (2011), pp. 86–94.

S.A.E. Kools, J.F. Moltmann, T. Knacker Estimating the use of veterinary medicines in the European union Regulatory Toxicology and Pharmacology, 50 (1) (2008), pp. 59–65.

A. Kot-Wasik, A. Wasik Determination of robenidine in animal feeds by liquid chromatography coupled with diode-array detection and mass spectrometry after accelerated solvent extraction Analytica Chimica Acta, 543 (1–2) (2005), pp. 46–51.

N. León, M. Roca, C. Igualada, C.P.B. Martins, A. Pastor, V. Yusá Wide-range screening of banned veterinary drugs in urine by ultra high liquid chromatography coupled to high-resolution mass spectrometry Journal of Chromatography A, 1258 (2012), pp. 55–65.

R.P. Lopes, T.E. De Freitas Passos, J.F. De Alkimim Filho, E.A. Vargas, D.V. Augusti *et al.* Development and validation of a method for the determination of sulfonamides in animal feed by modified QuEChERS and LC–MS/MS analysis Food Control, 28 (1) (2012), pp. 192–198.

A. Martínez-Villalba, L. Vaclavik, E. Moyano, M.T. Galceran, J. Hajslova Direct analysis in real time high-resolution mass spectrometry for high-throughput analysis of antiparasitic veterinary drugs in feed and food Rapid Communications in Mass Spectrometry, 27 (3) (2013), pp. 467–475.

J. Nácher-Mestre, M. Ibáñez, R. Serrano, J. Pérez-Sánchez, F. Hernández Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry Journal of Agricultural and Food Chemistry, 61 (9) (2013), pp. 2077–2087.

A.A.M. Stolker, P. Rutgers, E. Oosterink, J.J.P. Lasaroms, R.J.B. Peters, J.A. Van Rhijn *et al.* Comprehensive screening and quantification of veterinary drugs in milk using UPLC-ToF-MS Analytical and Bioanalytical Chemistry, 391 (6) (2008), pp. 2309–2322.

E. van der Heeft, Y.J.C. Bolck, B. Beumer, A.W.J.M. Nijrolder, A.A.M. Stolker, M.W.F. Nielen Full-scan accurate mass selectivity of ultra-performance liquid chromatography combined with time-of-flight and orbitrap mass spectrometry in hormone and veterinary drug residue analysis Journal of the American Society for Mass Spectrometry, 20 (3) (2009), pp. 451–463.

F. Van Holthoon, P.P.J. Mulder, E.O. Van Bennekom, H. Heskamp, T. Zuidema, H.J.A. Van Rhijn Quantitative analysis of penicillins in porcine tissues, milk and animal feed using

derivatisation with piperidine and stable isotope dilution liquid chromatography tandem mass spectrometry Analytical and Bioanalytical Chemistry, 396 (8) (2010), pp. 3027–3040.

M. Villar-Pulido, B. Gilbert-López, J.F. García-Reyes, N.R. Martos, A. Molina-Díaz Multiclass detection and quantitation of antibiotics and veterinary drugs in shrimps by fast liquid chromatography time-of-flight mass spectrometry Talanta, 85 (3) (2011), pp. 1419–1427.

U. Vincent, M. Chedin, S. Yasar, C. von Holst Determination of ionophore coccidiostats in feedingstuffs by liquid chromatography–tandem mass spectrometry. Part I. Application to targeted feed Journal of Pharmaceutical and Biomedical Analysis, 47 (4–5) (2008), pp. 750–757.

H. Xu, T. Wang, Q. Zhao, Q. Zeng, H. Wang, Y. Xu *et al.* Analysis of fluoroquinolones in animal feed based on microwave-assisted extraction by LC–MS–MS determination Chromatographia, 74 (3–4) (2011), pp. 267–274.

SUPPLEMENTARY INFORMATION

Qualitative screening of 116 veterinary drugs in feed by liquid chromatography–high resolution mass spectrometry: potential application to quantitative analysis

Table S1. Retention time (Rt), ionization mode, elemental composition, exact mass of (de)protonated molecule and elemental composition of the main fragment/adduct ions used for identification of the compounds.

Pharmaceutical	Rt (min)	Ion mode	Elemental composition	$[M+H]^+$ /$[M-H]^-$	Fragment ion 1	Fragment ion 2	Fragment ion 3	Fragment ion 4	Fragment ion 5
Stilbenes									
Dienestrol	11.2	-	C18H18O2	265.1229	C17H14O2	C16H12O2	C6H6O	C15H10O2	C12H12O
Diethylstilbestrol	11.9	-	C18H20O2	267.1385	C14H10O2	C16H16O2	C16H14O2	C15H11O2	C9H8O
Hexestrol	11.3	-	C18H22O2	269.1542	C9H12O	C9H11O	C9H10O	C8H8O	C7H7O
Steroids									
Δ1,4-Androstadiene-3,17-dione	9.6	+	C19H24O2	285.1855	-	-	-	-	-
16-β-hydroxystanozolol	10.5	+	C21H32N2O2	345.2542	C4H4N2	-	-	-	-
α-Boldenone	10.9	+	C19H26O2	287.2011	C19H24O	C10H14	C19H26O2Na	C19H26O2K	C8H8O
α-Nandrolone	11.0	+	C18H26O2	275.2011	C18H24O	C10H12	C18H22	C11H12	C7H8O
Androstenediol (AED)	12.9	+	C19H30O2	291.2324	-	-	-	-	-
β-Boldenone	10.2	+	C19H26O2	287.2011	C19H26O2Na	C19H24O	C10H14	C19H26O2K	C8H8O
β-Nandrolone	10.3	+	C18H26O2	275.2011	C18H24O	C10H12	C18H22	C11H12	C7H8O
Stanozolol	12.1	+	C21H32N2O	329.2593	C7H10	C4H4N2	-	-	-
Resorcylic acid lactones (RALs)									
α-Zeranol	10.5	-	C18H26O5	321.1702	C17H26O3	C17H26O4	C18H24O4	C17H24O2	C15H24O2
β-Zeranol	9.6	-	C18H26O5	321.1702	C17H26O3	C18H24O4	C17H26O4	C17H24O2	C15H24O2
Zearalanone (ZAN)	10.9	-	C18H24O5	319.1545	C18H22O4	C17H24O3	C14H18O3	C12H14O3	C10H12O2
β-Agonists									
Brombuterol	5.6	+	C12H18Br2N2O	364.9864	C12H16N2Br2	C8H8N2Br	C8H8N2Br2	C8H7N2	
Clenbuterol	4.9	+	C12H18Cl2N2O	277.0874	C8H7N2	C12H16N2Cl2	C8H8N2Cl2	C8H5NCl	C8H9N2Cl
Clenpenterol	5.9	+	C13H20Cl2N2O	291.1031	C13H18N2Cl2	C8H8N2Cl2	C8H8N2Cl	C8H5NCl	C7H6NCl
Hydroxymethyl clenbuterol	3.9	+	C12H18Cl2N2O2	293.0824	C12H16N2OCl2	C8H8N2Cl	C8H7N2	C8H8N2Cl2	-
Mabuterol	5.8	+	C13H18ClF3N2O	311.1138	C9H8N2F3Cl	C9H7N2F2Cl	C13H16N2F3Cl	C9H8N2F3	C8H6NF2Cl
Mapenterol	6.8	+	C14H20ClF3N2O	325.1295	C9H8N2F3Cl	C9H7N2F2Cl	C9H8N2F3	C14H18N2F3Cl	C9H6N2FCl
Ractopamine	4.3	+	C18H23NO3	302.1756	C8H8O	C7H6O	C18H21NO2	C10H13NO	C8H9NO
Nitromidazoles									
Chloramphenicol	6.3	+	C11H12Cl2N2O	323.0201	C11H10N2O4Cl2	C8H8N2O2	C11H11NaCl2N2O5	C8H8N	C10H8N2O3Cl2
		-	C11H12N2O5Cl2	321.0045	C10H11N2O4Cl	C9H9NO4	C7H7NO3	C9H7NO3	C10H10N2O5
Dimetridazole (DMZ)	2.8	+	C5H7N3O2	142.0617	C5H10N2O	C4H8N3	C5H9N2	C4H6N2	C5H10N2
Furaltadone	2.4	+	C13H16N4O6	325.1148	C5H9NO	C11H13N3O4	C7H13NO	-	-
Hydroxy Dimetridazole (HMMNI)	2.1	+	C5H7N3O3	158.0566	-	-	-	-	-
Hydroxyipronidazole (IPZOH)	4.4	+	C7H11N3O3	186.0879	C7H9N3O2	C7H9N2	C4H5N3O2	C6H6N2	C6H1N
Hydroxymetronidazole (MNZOH)	1.8	+	C6H9N3O4	188.0671	-	-	-	-	-

Table S1 (cont.). Retention time (Rt), ionization mode, elemental composition, exact mass of (de)protonated molecule and elemental composition of the main fragment/adduct ions used for identification of the compounds.

Pharmaceutical	Rt (min)	Ion mode	Elemental composition	$[M+H]^+$ /$[M-H]^-$	Fragment ion 1	Fragment ion 2	Fragment ion 3	Fragment ion 4	Fragment ion 5
Ipronidazole (IPZ)	5.4	+	C7H11N3O2	170.0930	-	-	-	-	-
Metronidazole (MNZ)	5.4	+	C6H9N3O3	172.0722	C6H11N2O	C5H9N3O	C6H10N2O	C6H11N3	C5H9N3
Ronidazole (RNZ)	12.1	-	C6H8N4O4	199.0467	C5H6N2O4	C3N2O2	C4H4N3	-	-
Amphenicols									
Amoxicillin	4.2	+	C16H19N3O5S	366.1123	-	-	-	-	-
Ampicillin	4.2	+	C16H19N3O4S	350.1174	C7H7N	C6H9NO2S	C10H7NO2	C8H7N	C4H3NOS
Cefaclor	3.9	+	C15H14N3O4SCl	368.0472	-	-	-	-	-
Cefadroxil	4.9	+	C16H17N3O5S	364.0967	-	-	-	-	-
Cefotaxim	4.0	+	C16H17N5O7S2	456.0647	C14H13N5O5S2	C12H13N5O2S2	C6H4N4OS	C7H6N2OS	C13H13N5O4S2
Ceftriaxone	11.3	+	C18H18N8O7S3	555.0539	-	-	-	-	-
Cefuroxime	9.0	+	C16H16N4O8S	425.0767	-	-	-	-	-
Cephalexin	3.9	+	C16H17N3O4S	348.1018	C6H7NO2S	C6H5NOS	C10H7NO2	C8H7N	C7H7N
Chlortetracycline	6.1	+	C22H23ClN2O8	479.1221	C22H20NO8Cl	C9H5O3Cl	-	-	-
Ciprofloxacin	4.2	+	C17H18N3O3F	332.1410	C17H16N3O2F	C12H7N2O2F	C14H13N2OF	C11H7N2OF	C16H18N3OF
Clarithromycin	10.3	+	C38H69NO13	748.4847	C30H55NO10	C8H15NO2	C29H51NO9	C6H13NO	C5H6O
Cloxacillin	9.4	+	C19H18ClN3O5S	436.0734	C6H9NO2S	C19H17NaClN3O5S	C9H4NOCl	C13H9N2O3Cl	C11H8NO2Cl
Dicloxacillin	10.0	+	C19H17Cl2N3O5S	470.0344	C13H8N2O3Cl2	C19H16NaCl2N3O5S	C14H5N3O3Cl2	C11H7NOCl2	C9H5NCl2
Doxycycline	7.2	+	C22H24N2O8	445.1611	-	-	-	-	-
Enrofloxacin	4.4	+	C19H22FN3O3	360.1723	C18H22N3OF	C14H13N2OF	C19H20N3O2F	-	-
Erythromycin A	9.3	+	C37H67NO13	734.4690	C8H15NO2	C37H65NO12	C6H13NO	C29H53NO10	-
Florfenicol	4.5	+	C12H14Cl2FNO4S	358.0083	C8H9NO2FCl	C6H10NCl	C11H6O3FCl	C8H8NO2F	
Florfenicol amine	1.2	+	C10H14FNO3S	248.0757	-	-	-	-	-
Flumequine	8.1	+	C14H12NO3F	262.0879	C11H4NO2F	C10H4NOF	C14H9NO2F	C14H11NO2F	-
Lincomycin	3.6	+	C18H34N2O6S	407.2216	C8H15N	-	-	-	-
Nalidixic acid	7.7	+	C12H12N2O3	233.0926	C10H6N2O2	C12H10N2O2	C9H6N2O	-	-
Norfloxacin	4.0	+	C16H18N3O3F	320.1410	C16H16N3O2F	C13H13N2OF	C15H18N3OF	C16H15N3O2	C12H7N2O2F
Ofloxacin	3.9	+	C18H20N3O4F	362.1516	C7H20N3O2F	C14H13N2O2F	C18H18N3O3F	C14H9N2O2F	C4H7N
Oxacillin	9.0	+	C19H19N3O5S	402.1124	C13H10N2O3	C6H9NO2S	C19H18NaN3O5S	C14H7N3O3	C11H10N3O3S
Oxolinic acid	6.4	+	C13H11NO5	262.0715	C13H9NO4	C11H5NO4	C10H5NO2	C9H5NO2	C11H4NO4
Oxytetracycline	4.5	+	C22H24N2O9	461.1560	C22H22N2O8	C22H19NO8	C22H21NO9	C22H17NO7	C20H12O8
Pefloxacin	3.9	+	C17H20FN3O3	334.1567	C17H18N3O2F	C13H13N2OF	C16H20N3OF	C11H9N2OF	C11H7N2OF
Penicillin G	7.9	+	C16H18N2O4S	335.1065	C10H9NO2	C6H9NO2S	C16H17NaN2O4S	C8H9N2O2S	C8H7NO2S
Pipedimic acid	3.3	+	C14H17N5O3	304.1409	C14H15N5O2	C11H12N4O	C10H6N4O2	C9H8N4O	C8H7N3O

Table S1 (Cont). Retention time (Rt), ionization mode, elemental composition, exact mass of (de)protonated molecule and elemental composition of the main fragment/adduct ions used for identification of the compounds.

Pharmaceutical	Rt (min)	Ion mode	Elemental composition	$[M+H]^+$ /$[M-H]^-$	Fragment ion 1	Fragment ion 2	Fragment ion 3	Fragment ion 4	Fragment ion 5
Piperacillin	7.9	+	C23H27N5O7S	518.1709	C14H15N5O7S	C6H10N2O2	C6H9NO2S	C23H26N5NaO7S	C17H18N4O5
Roxythromycin	10.5	+	C41H76N2O15	837.5324	C33H62N2O12	C8H15NO2	C5H6O	C35H60N2O12	C28H45N2O7
Sarafloxacin	4.9	+	C20H17F2N3O3	386.1316	C20H15N3O2F2	C20H11N2F	C19H17N3OF2	C17H20N3O2F	-
Sulfadoxine	4.8	+	C12H14N4O4S	311.0814	C6H5NO2S	C12H13N4O4SNa	C6H5NO	C6H7N3O2	C5H5N3O2
Sulfamethoxazole	4.4	+	C10H11N3O3S	254.0599	C6H5NO2S	C6H5N	C6H5NO	C4H6N2O	-
Tetracycline	4.4	+	C22H24N2O8	445.1611	C22H19NO7	C7H7NO3	-	-	-
Thiamphenicol	3.4	+	C12H15Cl2NO5S	356.0126	C11H8NOCl2	C11H8NOCl	C9H7O	-	-
		-	C12H15Cl2NO5S	353.9970	C8H10O3S	C8H8O	CH4O2S	C11H14NO4SCl	C12H13NO5S
Trimethoprim	3.6	+	C14H18N4O3	291.1457	C12H12N4O3	C14H15NO2	C12H13N4O	C13H14N4O3	C13H12N4O2
Avermectins									
Abamectine B1a	15.4	+	C48H71O14Na	895.4820	C48H72O14	-	-	-	-
Doramectine	16.8	+	C50H74O14	899.5157	C50H74O14Na	C50H74O14K	-	-	-
Emamectin B1a	13.5	+	C49H75NO13	886.5317	C7H11NO	C49H75NO13Na	C12H13	C19H25O3	-
Eprinomectin	14.9	+	C50H75NO14	914.5266	C50H75NO14Na	C6H9NO	C15H23NO5	C9H15NO3	C8H11NO2
Ivermectin	16.0	+	C48H74O14	875.5157	C48H73O14Na	-	-	-	-
Levamisole	2.8	+	C11H12N2S	205.0799	C10H11NS	C7H6	C7H6S	C10H8	-
Moxidectin	15.6	+	C37H53NO8	640.3849	C29H37NO6	C30H41NO7	C37H53NO8Na	C37H53NO8K	C29H41NO6
Coccidiostats									
Maduramicine	15.5	+	C47H79O17Na	939.5293	C46H77O14Na	C42H77O18Na	C37H56O8	-	-
Monensin	15.1	+	C36H62O11	671.4370	C36H61O11Na	C36H61O11K	C28H44O5	C36H59O10Na	-
		-	C36H62O11	669.4214	C35H58O10	C11H20O2	C5H10O2	C4H8O2	-
Narasin	16.1	+	C43H72O11	765.5153	C13H20O3	C23H32O4	C43H72O11Na	C42H68O10	C42H66O9
		-	C43H72O11	763.4996	C23H36O6	C20H36O5	C19H29O5	C14H24O4	C13H24O2
Robenidine	10.8	+	C15H13Cl2N5	334.0626	C8H4N3Cl	C7H7N2Cl	C7H5Cl	C8H7N4Cl	C7H6NCl
Salinomycin	15.7	+	C42H70O11	751.4996	C42H70O11Na	C42H70O11K	C42H68O10	C42H66O9	-
		-	C42H70O11	749.4840	C19H30O5	C35H60O11	C23H36O6	C19H34O5	-
Sedatives									
Acepromazine	8.8	+	C19H22N2OS	327.1531	C5H11N	C15H12NS	C13H9NS	C17H15NOS	C15H11NOS
Alprazolam	9.6	+	C17H13ClN4	309.0907	C16H11N3Cl	C17H13N4	C15H4N4	C14H8N2	C8H5N2Cl
Azaperol	5.0	+	C19H24FN3O	330.1982	C10H9F	C7H8N2	C19H22N3F	C12H14NF	C9H12N2
Azaperone	5.6	+	C19H22FN3O	328.1825	C10H9OF	C7H3OF	C7H10N2	C9H10N2	-
Carazolol	6.0	+	C18H22N2O2	299.1760	C15H11NO	C12H9NO	C6H13NO	C3H7NO	C13H9NO
Chlorpromazine	10.1	+	C17H19ClN2S	319.1036	C13H8NCl	C5H11N	C15H12NSCl	C13H8NSCl	C13H8NS

Table S1 (cont.). Retention time (Rt), ionization mode, elemental composition, exact mass of (de)protonated molecule and elemental composition of the main fragment/adduct ions used for identification of the compounds.

Pharmaceutical	Rt (min)	Ion mode	Elemental composition	$[M+H]^+$ /$[M-H]^-$	Fragment ion 1	Fragment ion 2	Fragment ion 3	Fragment ion 4	Fragment ion 5
Propionilpromazine (combelen)	9.8	+	C20H24N2OS	341.1688	C16H13NOS	C5H11N	C16H13NO	C18H17NOS	C3H7N
Non steroidal anti-inflammatory (NSAIDs)									
4-Acetylamino-antipyrine	3.6	+	C13H15N3O2	246.1242	C13H15N3O2	C13H13N3O	C11H13N3O	C7H5N	C10H10N2
4-amino-antipyrine	3.5	+	C11H13N3O	204.1137	C4H6N2	C7H5N	C4H6N2	C6H7N	C6H7N
4-formylamino-antipyrine	3.5	+	C12H13N3O2	232.1086	C12H12N3O2Na	C12H12N3O2K	C7H5N	C4H6N2	C6H7NO
Diclofenac	12.1	+	C14H11Cl2NO2	296.0245	C14H10NaCl2NO2	C14H9NOCl2	C13H8NCl	C13H9NCl	-
		-	C14H11Cl2NO2	294.0089	C13H9NCl2	C13H8NCl	-	-	-
Ibuprofen	12.4	-	C13H18O2	205.1229	C12H16	-	-	-	-
Ketoprofen	10.1	+	C16H14O3	255.1021	C16H13NaO3	C15H12O	C14H9O	C7H4O	-
		-	C16H14O3	253.0865	C15H12O	C13H8O2	-	-	-
Mefenamic acid	13.2	+	C15H15NO2	242.1181	C15H13NO	C14H10NO	C13H9N	-	-
Naproxen	10.4	+	C14H14O3	231.1021	C13H12O	C12H9O	C14H13NaO3	C12H8	C11H8
Oxyphenylbutazone	9.8	+	C19H20N2O3	325.1552	C19H19N2O3Na	C12H13NO2	C8H5NO2	C7H5NO	C11H13N
Phenylbutazone	11.4	+	C19H20N2O2	309.1603	C12H13NO	C11H13N	C7H5NO	C8H5NO	C6H5N
Salicylic acid	5.2	-	C7H6O3	137.0239	C6H4O	-	-	-	-
Corticoids									
Betamethasone o Dexamethasone	9.9	+	C22H29FO5	393.2077	C22H28O5	C22H26O4	C22H24O3	C22H22O2	C21H22O2
Flumethasone	9.5	+	C22H28F2O5	411.1983	C17H16O2	C14H15O2F	C8H8O	C9H10O	-
Methylprednisolone	9.9	+	C22H30O5	375.2171	C13H12O	C11H12O	C20H22O	C17H16O	C12H12O
Parasiticide									
Leucomalachite green	12.6	+	C23H26N2	331.2171	C14H13N	C16H18N2	C13H9N	C22H23N2	C17H20N2
Malachite green	9.7	+	C23H25ClN2	365.1785	C23H24N2	C22H20N2	C15H13N	C17H17N2	
Other pharmaceuticals									
Acetaminophen	2.2	+	C8H9NO2	152.0711	C6H7NO	C6H5N	C6H4O	C8H7NO	C8H7NO2
Atorvastatin	11.9	+	C33H35FN2O5	559.2608	C26H30NO4F	C20H18NF	C33H34NaFN2O5	C27H28NO5F	C19H14NF
		-	C33H35FN2O5	557.2452	C29H25N2O2F	C26H21N2OF	-	-	-
Bezafibrate	10.6	+	C19H20ClNO4	362.1159	C18H18NO2Cl	C15H14NO2Cl	C11H12O	C7H3OCl	C8H8O
		-	C19H20ClNO4	360.1003	C15H12NO2Cl	C7H4NOCl	C3H2NO2	-	-
Carbamazepine	8.1	+	C15H12N2O	237.1028	C14H10N	C14H11N	C13H8N	-	-
		-	C15H12N2O	235.0871	C14H12N	C14H11N	C14H10N	C13H9N	-
Enalapril	7.8	+	C20H28N2O5	377.2076	C14H19NO2	C14H19NO2	C11H13N	C17H22N2O3	C9H11N

Table S1 (Cont.). Retention time (Rt), ionization mode, elemental composition, exact mass of (de)protonated molecule and elemental composition of the main fragment/adduct ions used for identification of the compounds.

Pharmaceutical	Rt (min)	Ion mode	Elemental composition	$[M+H]^+$ /$[M-H]^-$	Fragment ion 1	Fragment ion 2	Fragment ion 3	Fragment ion 4	Fragment ion 5
Gemfibrozil	13.3	+	C15H22O3	251.1647	C15H21NaO3	C7H12O2	C15H20O2	-	-
	13.3	-	C15H22O3	249.1491	C8H8O	-	-	-	-
Irbesartan	10.6	+	C25H28N6O	429.2403	C14H10N2	C11H18N2O	C13H9N	C14H9N	-
		-	C25H28N6O	427.2246	C11H18N2O	C25H28N4O	-	-	-
Lorazepam	9.6	+	C15H10Cl2N2O2	321.0198	C14H8N2Cl2	C13H9N2Cl	C15H9NaCl2N2O2	C15H8N2OCl2	C14H7N2Cl
Olanzapine	3.7	+	C17H20N4S	313.1487	C14H13N3S	C12H8N2S	C16H15N3S	C11H5N2S	C11H7NS
Omeprazole	7.8	+	C17H19N3O3S	346.1225	C9H11NO2S	C9H11NO	C8H8N2O	C9H9NOS	C8H6NOS
Pantoprazole	8.1	+	C16H15F2N3O4S	384.0830	C7H7NO2	C8H9NO3S	C8H10NO2	-	-
Pravastatin	9.8	+	C23H36O7	425.2539	C23H35NaO7	C20H22O4	-	-	-
		-	C23H36O7	423.2383	C5H10O2	C18H24O4	C11H12	-	-
Valsartan	11.0	+	C24H29N5O3	436.2349	C24H28N5O3Na	C18H19N5	C14H10N4	C24H27N5O2	C24H29N3O3
		-	C24H29N5O3	434.2192	C14H12	C14H11	C19H21N5O2	-	-
Venlafaxine	7.0	+	C17H27NO2	278.2120	C17H25NO	C8H8O	C15H18O	-	-

Molecular formulae of the fragment ions differs ±1 hydrogen from the real one, depending on the ionization mode

Table S2. Validation results obtained after analysis of ten animal feed samples spiked at 0.02 and 0.2 mg kg^{-1}. Screening detection limits (SDL) and limits of identification (LOI) for each matrix, and SDL of the method (all in mg kg^{-1}).

Pharmaceutical	Poultry (n=2)		Bovine (n=2)		Rabbit (n=2)		Pork (n=2)		Goat (n=2)	
	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI
Stilbenes										
Dienestrol	0.2	0.2	0.2	-	-	-	0.2	-	-	-
Diethylstilbestrol	-	-	-	-	-	-	-	-	-	-
Hexestrol	0.2	0.2	-	-	0.2	-	0.2	0.2	-	-
Steroids										
Δ1,4-Androstadiene-3,17-dione (Boldione)[a]	0.02	-	0.2	-	0.2	-	-	-	-	-
16-β-hydroxystanozolol[b]	-	-	-	-	-	-	-	-	-	-
α-Boldenone	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.2
α-Nandrolone	0.02	0.02	0.02	0.2	0.02	-	0.02	0.02	0.02	0.02
Androstenediol (AED)[a]	0.02	-	0.02	-	-	-	0.02	-	0.02	-
β-Boldenone	0.02	0.02	0.02	-	0.02	0.2	0.02	0.02	0.2	0.2
β-Nandrolone	0.02	0.2	0.02	0.2	0.02	0.02	0.2	0.2	0.02	0.02
Stanozolol	0.02	0.2	0.02	-	0.02	0.02	0.02	-	0.02	-
RALs										
α-Zeranol	0.02	0.2	0.2	0.2	-	-	0.02	0.2	0.2	0.2
β-Zeranol	0.02	0.2	0.2	0.2	0.02	0.2	0.02	0.2	0.2	-
Zearalanone (ZAN)	-	-	-	-	0.2	-	-	-	-	-
β-Agonists										
Brombuterol	0.02	0.02	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02
Clenbuterol	0.02	0.02	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02
Clenpenterol	0.02	0.02	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02
Hydroxymethyl clenbuterol	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Mabuterol	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Mapenterol	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Ractopamine	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.2
Nitromidazoles										
Chloramphenicol	0.02	0.2	0.02	0.2	0.02	0.2	0.02	0.2	0.02	0.2
Dimetridazole (DMZ)	0.02	-	0.02	-	0.02	0.2	0.02	0.2	0.02	0.02
Furaltadone	0.02	0.2	0.02	-	0.2	0.2	0.02	0.2	0.02	0.2
OH Dimetridazole (HMMNI) [a]	-	-	0.02	-	-	-	-	-	-	-
Hydroxyipronidazole (IPZOH)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Hydroxymetronidazole (MNZOH)[a]	0.2	-	0.2	-	0.2	-	0.2	-	-	-
Ipronidazole (IPZ)[a]	0.02	-	0.2	-	0.2	-	0.02	-	0.02	-
Metronidazole (MNZ)	-	-	-	-	0.02	-	-	-	-	-
Ronidazole (RNZ)	0.2	-	0.2	-	-	-	0.2	-	-	-
Amphenicols										
Amoxicillin[a]	-	-	-	-	-	-	-	-	-	-
Ampicillin	0.02	0.2	0.2	0.2	0.2	-	0.2	0.2	0.2	0.2

Table S2 (Cont.). Validation results obtained after analysis of ten animal feed samples spiked at 0.02 and 0.2 mg kg^{-1}. Screening detection limits (SDL) and limits of identification (LOI) for each matrix, and SDL of the method (all in mg kg^{-1}).

Pharmaceutical	Poultry (n=2)		Bovine (n=2)		Rabbit (n=2)		Pork (n=2)		Goat (n=2)	
	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI
Cefaclor[a]	-	-	-	-	-	-	-	-	-	-
Cefadroxil[a]	-	-	-	-	-	-	-	-	-	-
Cefotaxim	0.02	0.2	0.2	0.2	0.2	0.2	0.02	0.02	0.2	0.2
Ceftriaxone[a]	-	-	0.2	-	-	-	-	-	-	-
Cefuroxime[a]	-	-	0.2	-	0.2	-	-	-	-	-
Cephalexin	-	-	-	-	-	-	-	-	-	-
Chlortetracycline	-	-	0.2	0.2	0.2	-	0.2	-	0.2	-
Ciprofloxacin	0.02	0.2	0.2	0.2	0.2	0.2	0.02	0.02	0.2	0.2
Clarithromycin	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Cloxacillin	0.2	0.2	0.2	0.2	0.02	0.2	0.2	0.2	0.2	0.2
Dicloxacillin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-
Doxycycline[a]	-	-	-	-	0.02	-	0.02	-	0.02	-
Enrofloxacin	0.02	0.2	0.02	0.2	0.2	0.2	0.02	0.2	0.02	0.2
Erythromycin A	0.2	0.2	0.2	0.2	0.2	-	0.2	-	0.2	-
Florfenicol	0.2	-	0.2	0.2	0.02	0.02	0.2	0.2	0.2	0.2
Florfenicol amine[a]	0.2	-	0.2	-	-	-	0.2	-	-	-
Flumequine	0.02	0.2	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Lincomycin[b]	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Nalidixic acid	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Norfloxacin	0.02	0.2	0.2	0.2	0.02	0.2	0.02	0.02	0.2	0.2
Ofloxacin	0.02	0.2	0.2	0.2	0.02	0.2	0.02	0.02	0.02	0.2
Oxacillin	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-
Oxolinic acid	0.02	0.02	0.2	0.2	0.02	0.2	0.02	0.02	0.02	0.2
Oxytetracycline	-	-	0.2	0.2	0.02	0.02	0.02	0.02	-	-
Pefloxacin	0.02	0.02	0.02	0.2	0.02	0.2	0.02	0.2	0.02	0.2
Penicillin G	0.2	0.2	0.02	0.2	0.02	0.2	0.2	0.2	0.2	0.2
Pipedimic acid	0.2	0.2	0.2	0.2	0.02	0.2	0.2	0.2	0.02	0.2
Piperacillin	0.2	0.2	0.02	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Roxythromycin	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Sarafloxacin	0.02	0.2	0.02	0.2	0.2	-	0.02	0.2	0.2	0.2
Sulfadoxine	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Sulfamethoxazole	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.2	0.02	0.2
Tetracycline	-	-	0.2	-	-	-	0.02	0.02	-	-
Thiamphenicol	0.02	-	0.2	-	0.02	-	0.02	0.2	0.02	-
Trimethoprim	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Avermectins										
Abamectine B1a[b]	-	-	-	-	-	-	-	-	-	-
Doramectine	-	-	-	-	-	-	-	-	-	-
Emamectin B1a	0.02	0.02	0.02	0.2	0.02	0.02	0.2	0.2	0.02	0.2
Eprinomectin	0.2	-	0.2	-	0.2	-	0.2	0.2	0.2	0.2

Table S2 (Cont.). Validation results obtained after analysis of ten animal feed samples spiked at 0.02 and 0.2 mg kg^{-1}. Screening detection limits (SDL) and limits of identification (LOI) for each matrix, and SDL of the method (all in mg kg^{-1}).

Pharmaceutical	Poultry (n=2)		Bovine (n=2)		Rabbit (n=2)		Pork (n=2)		Goat (n=2)	
	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI
Ivermectin[b]	0.2	-	-	-	-	-	0.2	-	-	-
Levamisole	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.2
Moxidectin	0.2	0.2	0.2	-	-	-	0.2	0.2	0.2	0.2
Coccidiostats										
Maduramicine	0.02	0.2	0.02	0.2	0.02	0.2	0.02	0.2	0.02	0.2
Monensin	0.02	0.02	0.02	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Narasin	0.02	0.2	0.02	0.2	0.02	0.2	0.02	-	0.02	0.2
Robenidine	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.2
Salinomycin	0.02	0.02	0.2	0.2	0.2	0.2	0.2	-	0.2	-
Sedatives										
Acepromazine	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.2
Alprazolam	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.2
Azaperol	0.02	0.02	0.02	0.02	0.02	0.2	0.02	0.02	0.02	-
Azaperone	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Carazolol	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Chlorpromazine	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Propionilpromazine (combelen)	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
NSAIDs										
4-acetylamino-antipyrine	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
4-amino-antipyrine	0.2	0.2	0.02	-	0.2	-	0.02	-	-	-
4-formylamino-antipyrine	0.02	0.2	0.02	0.2	0.2	0.2	0.02	0.02	0.02	0.02
Diclofenac	0.2	0.2	0.2	0.2	0.2	0.2	0.02	0.02	0.2	0.2
Ibuprofen[b]	-	-	-	-	-	-	0.2	0.2	-	-
Ketoprofen	0.02	0.02	0.02	0.2	0.02	0.2	0.02	0.02	0.02	0.2
Mefenamic acid	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Naproxen	0.2	0.2	0.2	0.2	0.02	0.2	0.2	0.2	0.02	-
Oxyphenylbutazone	0.02	0.2	0.2	0.2	0.2	-	0.02	0.2	0.2	-
Phenylbutazone	0.2	0.2	0.2	0.2	0.2	-	0.02	0.2	-	-
Salicylic acid[b]	0.02	-	0.02	-	0.02	-	0.02	-	0.02	0.2
Corticoids										
Betamethasone/Dexamethasone	0.2	0.2	0.2	0.2	0.2	-	0.2	0.2	0.2	0.2
Flumethasone	0.2	0.2	0.2	-	0.2	0.2	0.2	0.2	0.2	-
Methylprednisolone	-	-	0.2	-	-	-	0.2	0.2	-	-
Parasiticide										
Leucomalachite green	0.02	0.02	-	-	-	-	0.2	0.2	0.2	0.2
Malachite green	0.02	0.02	0.02	0.02	0.02	0.2	0.2	0.2	0.02	0.02

Table S2 (Cont.). Validation results obtained after analysis of ten animal feed samples spiked at 0.02 and 0.2 mg kg^{-1}. Screening detection limits (SDL) and limits of identification (LOI) for each matrix, and SDL of the method (all in mg kg^{-1}).

Pharmaceutical	Poultry (n=2)		Bovine (n=2)		Rabbit (n=2)		Pork (n=2)		Goat (n=2)	
	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI	SDL	LOI
Other pharmaceuticals										
Acetaminophen	0.2	-	0.2	-	0.2	-	0.2	-	-	-
Atorvastatin	0.2	0.2	0.2	0.2	0.2	-	0.2	0.2	0.2	0.2
Bezafibrate	0.02	0.2	0.02	0.2	0.2	0.2	0.02	-	0.02	-
Carbamazepine	0.02	0.02	0.02	0.2	0.02	0.02	0.02	-	0.02	0.02
Enalapril	0.02	0.02	0.02	0.2	0.02	0.2	0.02	0.02	0.02	0.02
Gemfibrozil	0.02	0.02	0.2	0.2	0.2[c]	0.2[c]	0.2	0.2	0.2[c]	0.2[c]
Irbesartan	0.02	0.02	0.02	0.2	0.02	0.02	0.02	0.02	0.02	0.02
Lorazepam	0.2	0.2	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Olanzapine	0.02	0.02	0.2	0.2	0.2	0.2	0.02	0.02	0.2	0.2
Omeprazole	-	-	-	-	-	-	-	-	-	-
Pantoprazole	0.2	0.2	0.2	0.2	-	-	0.2	-	-	-
Pravastatin	0.02	0.2	0.2	0.2	0.02	0.2	0.02	0.02	0.02	0.2
Valsartan	0.02	0.02	0.02	0.02	0.2	0.2	0.02	0.02	0.02	0.02
Venlafaxine	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02

[a] Compound without fragment ions
[b] Compound with only one fragment ion
[c] Found in negative ionization mode

Table S3. Recovery experiments for the compounds detected in feed samples.

Compound	Ionization mode	Recovery (RSD) (both in %)						Isotopically-labelled internal standard (ILIS)
		Bovine (n=3)		Poultry (n=3)		Pork (n=3)		
		0.2 mg kg^{-1}	2 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}	0.2 mg kg^{-1}	2 mg kg^{-1}	
β-Nandrolone	+	94 (14)	144 (3)	76 (15)	123 (3)	93 (5)	110 (4)	Fenilbutazone-d_{10}
Robenidine	+	93 (6)	94 (3)	61 (3)	90 (3)	70 (1)	98 (6)	Robenidine-d_8
Chlortetracycline	+	-	-	-	-	-	81 (22)	Amphetamine-d_6
Oxytetracycline	+	-	-	-	-	-	108 (9)	Amphetamine-d_6
Tetracycline	+	-	-	-	-	-	101 (4)	Amphetamine-d_6
Doxicycline	+	-	-	-	-	-	115 (11)	Amphetamine-d_6
Lincomycin	+	105 (1)	121 (2)	92 (0)	104 (2)	-	-	Carbamazepine epoxide-d_{10}
Trimethoprim	+	57 (5)	120 (2)	51 (0)	96 (6)	33 (7)	85 (2)	Fenilbutazone-d_{10}
Florfenicol	+	73 (8)	58 (4)	71 (9)	98 (2)	59 (10)	81 (10)	Benzoylecgonine-d_3
Acid salicylic	-	-	108 (10)	-	39 (19)	-	132 (7)	4,4'dinitrocarbanilide-d_8

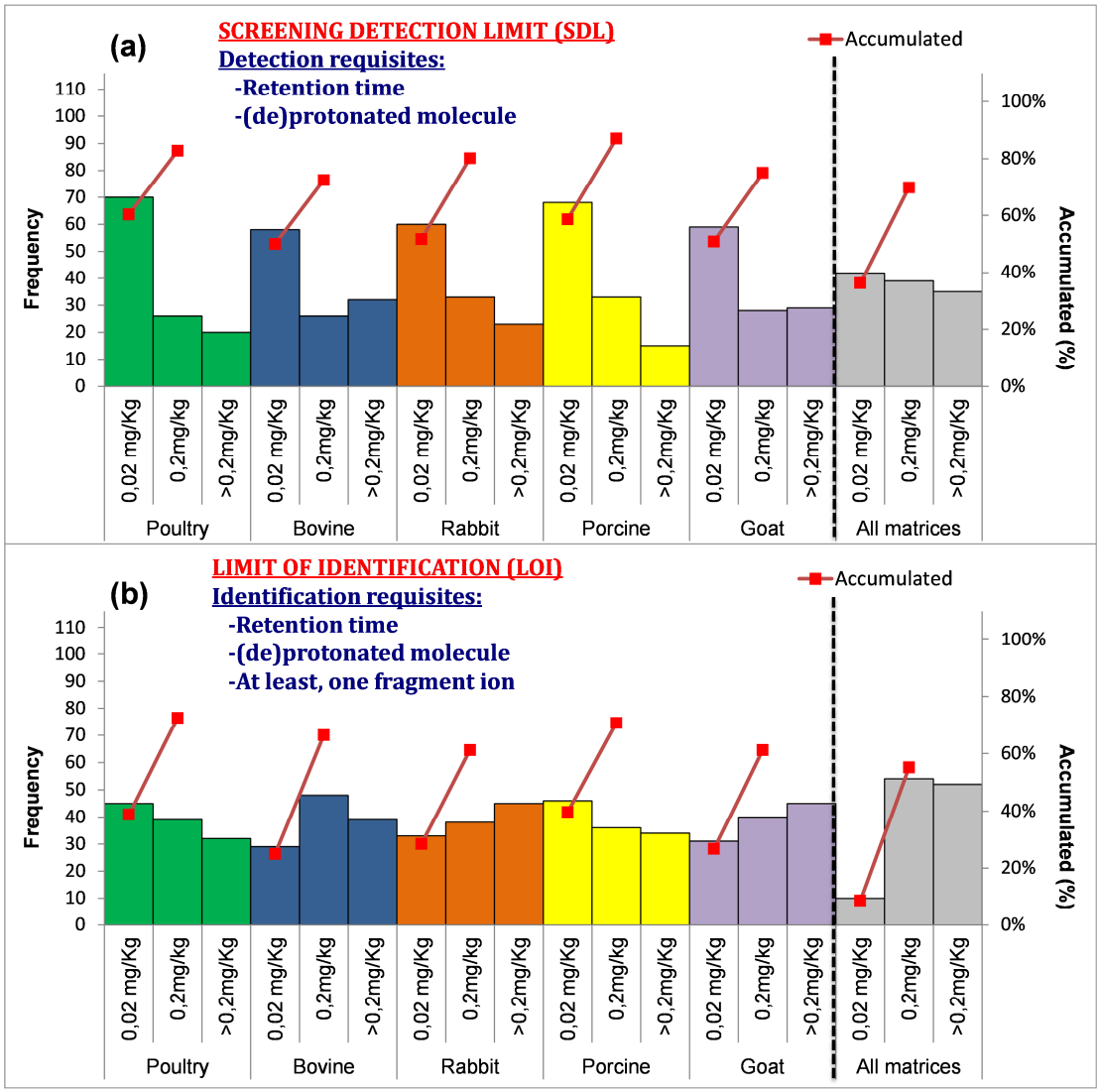

Figure S1. Number of analytes (a) detected and (b) identified at 0.02 and 0.2 mg kg^{-1} in each feed matrix.

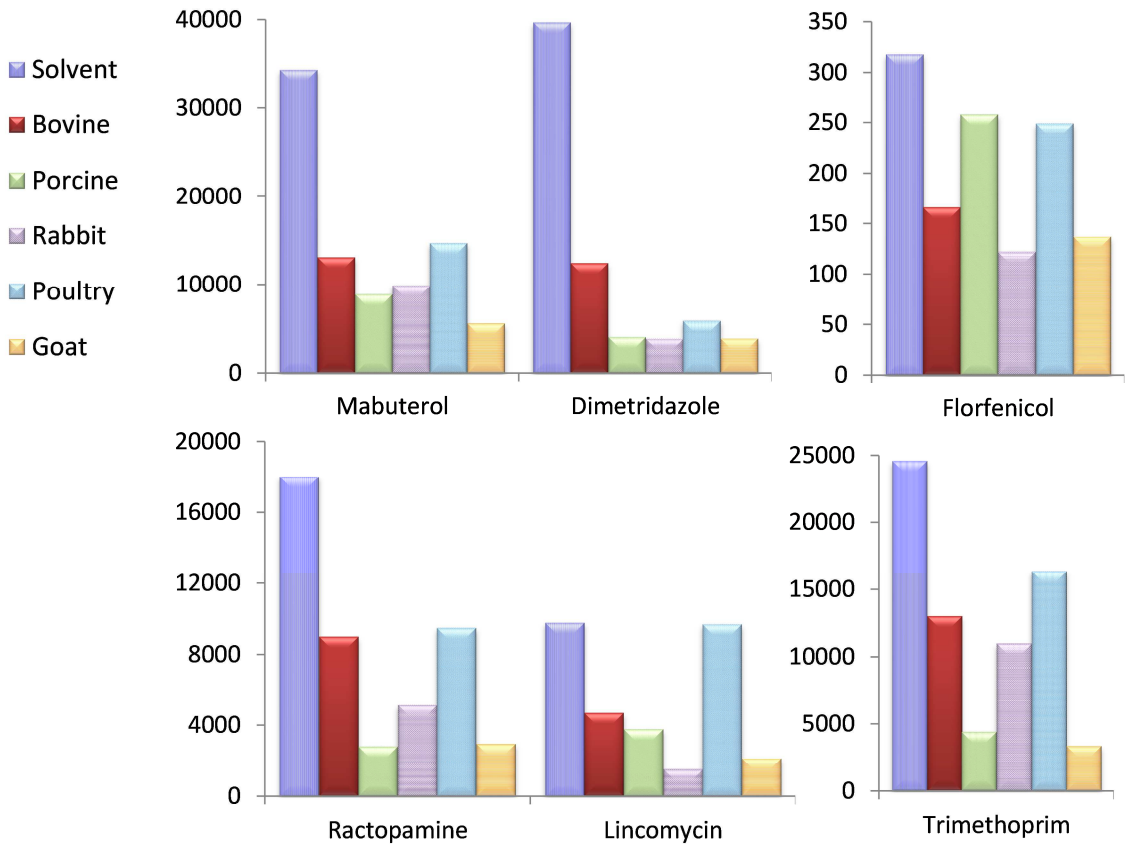

Figure S2. Absolute response for six pharmaceuticals in solvent and in five feed matrices spiked at 0.2 mg kg^{-1}.

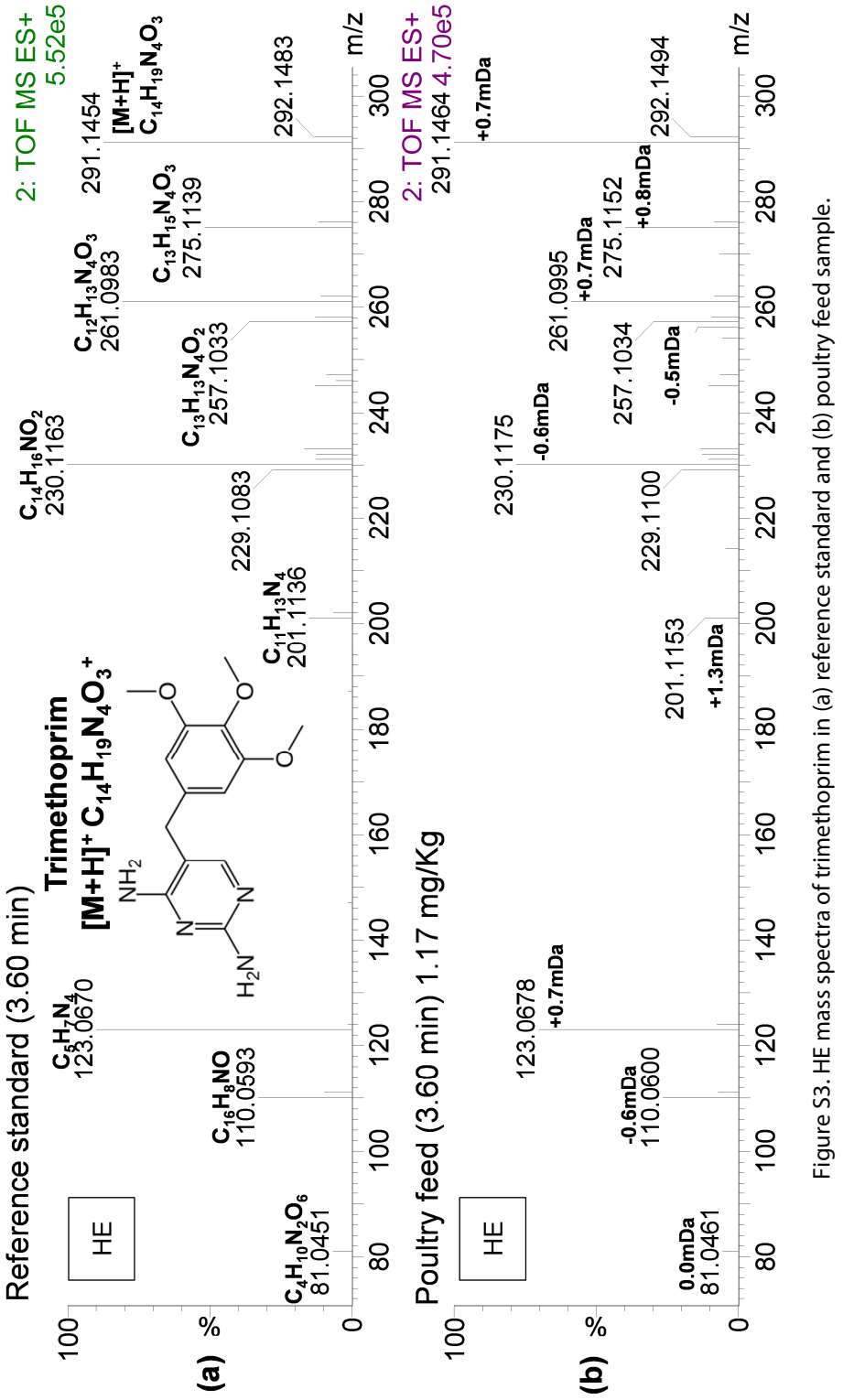

Figure S3. HE mass spectra of trimethoprim in (a) reference standard and (b) poultry feed sample.

6.3 Discusión de los resultados

La primera tarea que se llevó a cabo fue la creación de una base de datos con los 116 fármacos, humanos y veterinarios, objeto de estudio. La selección de dichos fármacos se hizo en base a su potencial presencia en los piensos y a datos previamente reportados en la bibliografía.

Inicialmente, se adquirieron los patrones de referencia y se inyectaron en el sistema UHPLC-ESI-QTOF MS en modo MS^E, con el fin de conocer su tiempo de retención así como sus iones fragmento característicos en masa exacta. En la **Figura 6.2** se muestra un ejemplo para la acepromazina (fármaco veterinario sedante), donde el espectro LE proporciona información acerca de la molécula protonada $[M+H]^+$, mientras que en el espectro HE se observan hasta 7 iones fragmento a los que se les asignó la fórmula empírica y se propuso una posible estructura química. Tanto el tiempo de retención como los iones fragmento son necesarios para la posterior confirmación de los positivos.

Siguiendo esta metodología para todos los compuestos, se creó la base de datos detallada en la *Tabla S1* del *Artículo Científico 7*. Para cada fármaco se incluye el Rt, el modo en que se ioniza preferentemente, la masa exacta de la molécula (des)protonada (según corresponda) y la composición elemental del compuesto y de sus iones fragmento. La mayor parte de los compuestos se determinaron en modo de ionización positivo, tan sólo nueve presentaron ionización en modo negativo y catorce presentan señal en ambos modos.

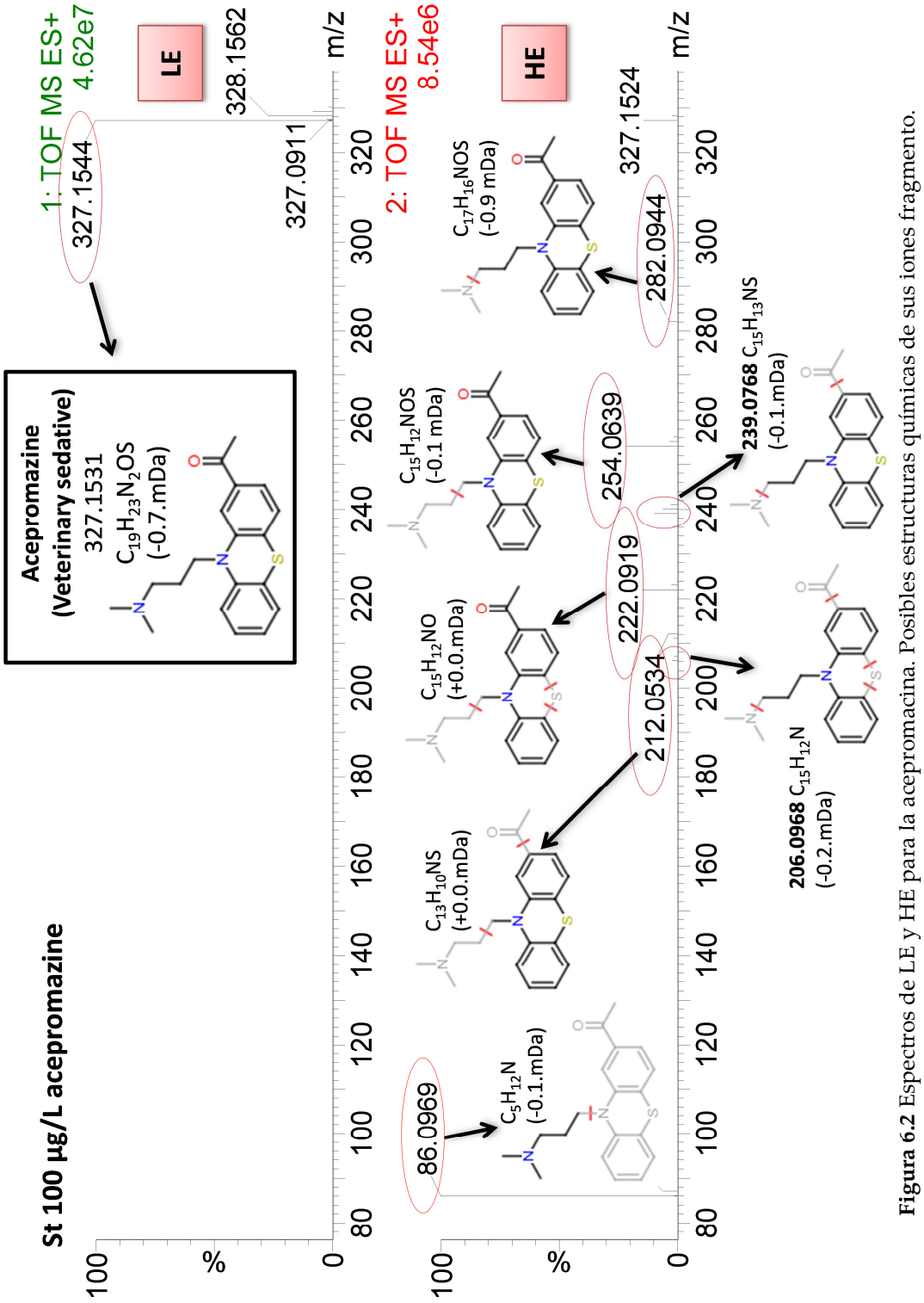

Figura 6.2 Espectros de LE y HE para la acepromacina. Posibles estructuras químicas de sus iones fragmento.

En un análisis tipo *screening* raramente se alcanza la situación ideal y óptima para todos los compuestos, por lo que es necesario llegar a un compromiso entre las condiciones de extracción, de separación cromatográfica y, finalmente, de detección por MS, para poder detectar satisfactoriamente y con la mayor sensibilidad posible el mayor número de analitos.

En el *Artículo Científico 7* se aplicaron las condiciones cromatográficas (columna, fases móviles y gradiente) y espectrométricas (adquisición en MS^E) utilizadas en trabajos previos de nuestro grupo de investigación (Nácher-Mestre, 2013; Díaz, 2013). Sin embargo, se trabajó con detalle la ampliación de la base de datos general que el grupo está creando, añadiendo fármacos de uso veterinario.

Las muestras de pienso son, por lo general, matrices muy complejas (Nácher-Mestre, 2013; Kaufmann, 2011; Mol, 2008; Aguilera-Luiz, 2013), por lo que el tratamiento de muestra es una etapa importante a optimizar. En el presente trabajo se llevó a cabo una extracción con acetonitrilo 1% acidificado con HCOOH. Tras ello, las muestras se analizaron mediante UHPLC-QTOF MS. El método desarrollado fue cualitativamente validado a los niveles de concentración 0.02 y 0.2 mg/Kg en cinco tipos de pienso (bovino, cunícola, avícola, caprino y porcino).

Los dos parámetros principales que se estudiaron en la validación fueron:
SDL: Un compuestos se consideró detectado cuando se observó su molécula (des)protonada (errores de masa menores de ±2 mDa) y el tiempo de retención fue consistente con el del patrón de referencia (± 2.5% de tolerancia del Rt).

LOI: Un compuesto se consideró identificado cuando además del cumplimiento del Rt, se observaron dos iones a la masa exacta, con errores de masa aceptables.

Las *Tablas 1* y *S2* del *Artículo Científico 7* muestran los valores de SDL y LOI para los fármacos estudiados en las cinco matrices analizadas. La mayoría de los compuestos fueron detectados al nivel de 0.02 mg/Kg, utilizando generalmente la molécula (des)protonada. Sin embargo, la identificación, con dos iones, a dicho nivel fue más problemática especialmente en los piensos caprino y bovino.

Como ya se ha apuntado anteriormente, los piensos son matrices muy complejas, observándose grandes diferencias entre los distintos tipos de piensos, debido, posiblemente, a sus diferentes contenidos en grasa. La **Figura 6.3** muestra un ejemplo para el β-antagonista mabuterol, en la validación de las cinco matrices objeto de estudio. En la figura se muestran los cromatogramas nw-XICs a *m*/*z* 311.1138 para las cinco matrices de pienso fortificadas al nivel bajo, 0.02 mg/Kg. Como puede verse, se observaron grandes variaciones en la señal según el tipo de matriz, mostrando en todos los casos supresión de la señal al ser comparados con el patrón de referencia en solvente. El pienso de bovino fue la matriz que presentó mayor efecto matriz, con una notable supresión de la ionización.

Finalmente, el método se aplicó a un total de 22 muestras de los cinco tipos de pienso validados (12 bovino, 3 cunícola, 2 avícola, 2 caprino y 3 porcino). Para ello, se utilizó una base de datos ampliada, conteniendo unos 650 fármacos (116 de ellos con patrón de referencia). Tras procesar los datos con el software ChromaLynx XS™ se pudieron detectar e identificar varios antibióticos, como la trimetoprima, la regulada robenidina, o los prohibidos α y β-nandrolona. También estuvieron presentes amfenicoles como el florfenicol o la lincomicina (ejemplos de estos positivos se muestran en las *Figuras 1-3* del *Artículo Científico 7*).

En una segunda etapa se llevó a cabo una evaluación semi-cuantitativa de los positivos encontrados en los piensos analizados. Para ello, una vez analizadas las

muestras, se fortificaron por triplicado tres piensos de diferente matriz (bovino, avícola y porcino) con los fármacos detectados a 0.2 y 2.0 mg/Kg. Se evaluó la linealidad del método, obteniendo coeficientes de correlación superiores al 0.99 en el intervalo estudiado. Por lo que se refiere a la precisión y exactitud, los ensayos de recuperación indicaron de nuevo que el efecto matriz experimentado por los analitos variaba notablemente según la composición del pienso. Por ello, se decidió utilizar compuestos marcados isotópicamente, para corregir y compensar los posibles errores producidos durante el proceso y/o efectos matriz inesperados (para más detalles ver el *Artículo Científico 7).* El hecho de disponer de pocos patrones marcados fue una limitación del método. Este estudio preliminar indicó que QTOF MS, además de ser una técnica muy poderosa para el amplio *screening* de fármacos en piensos, puede ser también una técnica adecuada para el análisis cuantitativo. Sin duda, serán necesarios más trabajos para demostrar si QTOF MS puede competir, en condiciones semejantes de sensibilidad y selectividad, con LC-MS/MS con triple cuadrupolo para el análisis cuantitativo en este campo.

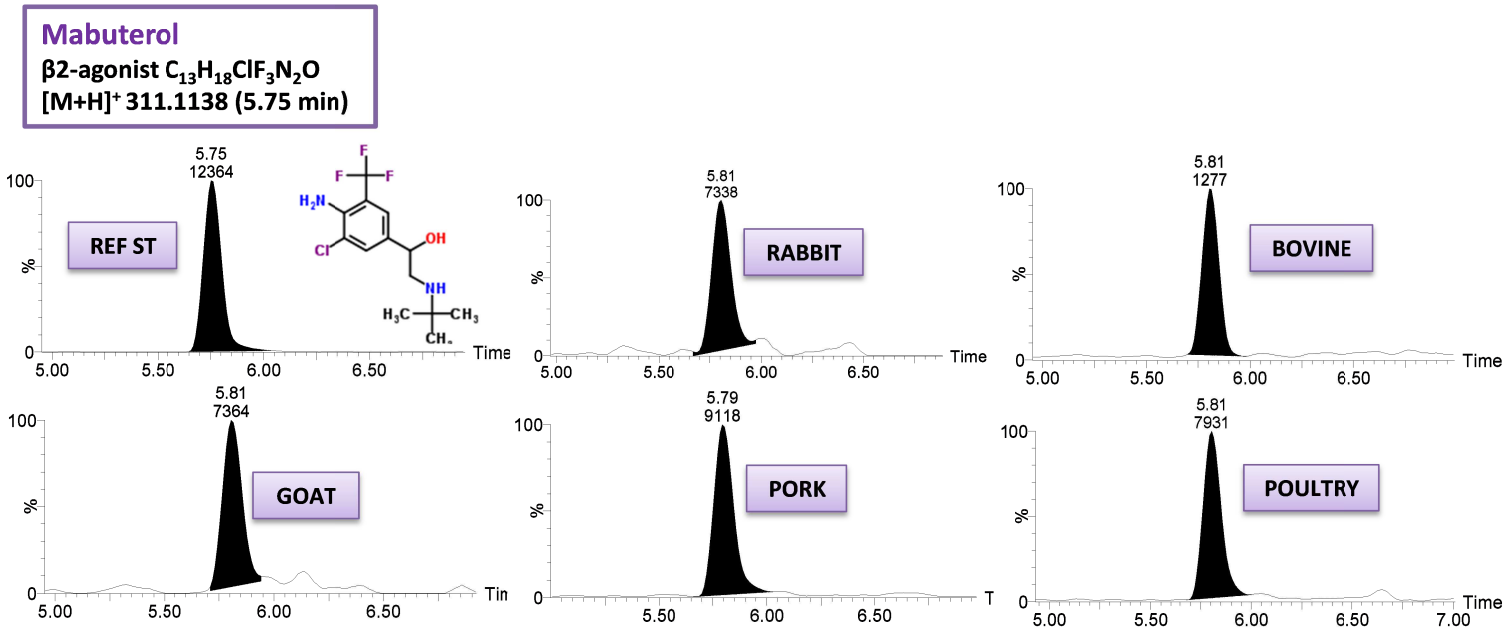

Figura 6.3 Nw-XICs de la molécula protonada del mabuterol en patrón de referencia y en los cinco tipos de matriz de pienso estudiados, fortificados a 0.02 mg/Kg.

CAPÍTULO 7

BIBLIOGRAFÍA

Bibliografía

Aguilera-Luiz, M.M., Romero-González, R., Plaza-Bolaños, P., Martínez Vidal, J.L., Garrido Frenich, A., 2013. Wide-scope analysis of veterinary drug and pesticide residues in animal feed by liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry. Anal. Bioanal. Chem. 1–11.

Almeida, B., Oehmen, A., Marques, R., Brito, D., Carvalho, G., Barreto Crespo, M.T., 2013. Modelling the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) by activated sludge and a pure culture. Bioresour. Technol. 133, 31–37.

Antonelli, M., Mezzanotte, V., Nurizzo, C., 2008. Wastewater disinfection by UV irradiation: Short and long-term efficiency. Environ. Eng. Sci. 25, 363–373.

Bagnati, R., Davoli, E., 2011. Illicit Drugs in the Environment, Illicit Drugs in the Environment: Occurrence, Analysis, and Fate Using Mass Spectrometry. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Baker, D.R., Kasprzyk-Hordern, B., 2013. Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: new developments. Sci. Total Environ. 454-455, 442–56.

Berset, J.-D., Brenneisen, R., Mathieu, C., 2010. Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography - Electrospray tandem mass spectrometry (HPLC-MS/MS). Chemosphere 81, 859–866.

Bijlsma, L., Sancho, J.V., Pitarch, E., Ibáñez, M., Hernández, F., 2009. Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater. J. Chromatogr. A 1216, 3078–3089.

Bijlsma, L., Beltrán, E., Boix, C., Sancho, J.V., Hernández, F., 2014. Improvements in analytical methodology for the determination of frequently consumed illicit drugs in urban wastewater. Anal. Bioanal. Chem. 406, 4261-4272.

Boleda, M.R., Galceran, M.T., Ventura, F., 2007. Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1175, 38–48.

Boleda, M.R., Galceran, M.T., Ventura, F., 2009. Monitoring of opiates, cannabinoids and their metabolites in wastewater, surface water and finished water in Catalonia, Spain. Water Res. 43, 1126–1136.

Boleda, M.R., Galceran, M.T., Ventura, F., 2011. Behavior of pharmaceuticals and drugs of abuse in a drinking water treatment plant (DWTP) using combined conventional and ultrafiltration and reverse osmosis (UF/RO) treatments. Environ. Pollut. 159, 1584–1591.

Buchberger, W.W., 2011. Current approaches to trace analysis of pharmaceuticals and personal care products in the environment. J. Chromatogr. A 1218, 603–18.

Castiglioni, S., Bijlsma, L., Covaci, A., Emke, E., Hernández, F., Reid, M., Ort, C., Thomas, K. V, van Nuijs, A.L.N., de Voogt, P., Zuccato, E., 2013. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers. Environ. Sci. Technol. 47, 1452–60.

Castiglioni, S., Zuccato, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2008. Mass spectrometric analysis of illicit drugs in wastewater and surface water. Mass Spectrom. Rev. 27, 378–94.

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R., Bagnati, R., 2006. Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. Anal. Chem. 78, 8421–8429.

Catalán Lafuente José. Química del agua MBH (Bellisco), 1969.

Celiz, M.D., Tso, J., Aga, D.S., 2009. Pharmaceutical metabolites in the environment: analytical challenges and ecological risks. Environ. Toxicol. Chem. 28, 2473–84.

Díaz, R., Ibáñez, M., Sancho, J.V., Hernández, F., 2013. Qualitative validation of a liquid chromatography-quadrupole-time of flight mass spectrometry screening method for organic pollutants in waters. J. Chromatogr. A 1276, 47–57.

Directiva del Consejo 96/23/CE; DIRECTIVA 96/23/CE DEL CONSEJO de 29 de abril de 1996 relativa las medidas de control aplicables respecto de determinadas sustancias y sus residuos en los animales vivos y sus productos y por la que se derogan las Directivas 85/358/CEE y 86/469/CEE y las Decisiones 89/187/CEE y 91/664/CEE.

Directiva 2004/28/CE; DIRECTIVA 2004/28/CE DEL PARLAMENTO EUROPEO Y DEL CONSEJO de 31 de marzo de 2004 que modifica la directiva 2001/81/CE por la que se establece un código comunitario sobre medicamentos veterinarios.

Directiva 2002/657/CE; Decisión de la Comisión de 12 de agosto de 2002 por la que se aplica la Directiva 96/23/CE del Consejo en cuanto al funcionamiento de los métodos analíticos y la interpretación de los resultados.

Directiva 2013/39/UE del Parlamento Europeo y del Consejo de 12 de agosto de 2013 por la que se modifican las Directivas 2000/60/CE y 2008/105/CE en cuanto a las sustancias prioritarias en el ámbito de la política de aguas.

Dorival-García, N., Zafra-Gómez, A., Navalón, A., González-López, J., Hontoria, E., Vílchez, J.L., 2013. Removal and degradation characteristics of quinolone antibiotics in laboratory-scale activated sludge reactors under aerobic, nitrifying and anoxic conditions. J. Environ. Manage. 120, 75–83.

EMCDDA, 2014 European Drug Report 2014: Trends and developments, Lisbon, May 2014 file:///C:/Users/cboix/Downloads/TDAT14001ESN%20(1).pdf.

Escher B. I., Fenner K., 2011. Recent advances in environmental risk assessment of transformation products. Environmental science & technology 45 (9) p. 3835-47.

Fabritius, M., Staub, C., Mangin, P., Giroud, C., 2012. Distribution of free and conjugated cannabinoids in human bile samples. Forensic Sci. Int. 223, 114–8.

Farré, M. l., Pérez, S., Kantiani, L., Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC - Trends Anal. Chem. 27, 991–1007.

Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011a. Pharmaceutical residues in environmental waters and wastewater: Current state of knowledge and future research. Anal. Bioanal. Chem. 399, 251–275.

Fatta-Kassinos, D., Vasquez, M.I., Kümmerer, K., 2011b. Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes - Degradation, elucidation of byproducts and assessment of their biological potency. Chemosphere 85, 693–709.

Ferrando-Climent, L., Collado, N., Buttiglieri, G., Gros, M., Rodriguez-Roda, I., Rodriguez-Mozaz, S., Barceló, D., 2012. Comprehensive study of ibuprofen and its metabolites in activated sludge batch experiments and aquatic environment. Sci. Total Environ. 438, 404–413.

Gómez, M.J., Malato, O., Ferrer, I., Agüera, A., Fernández-Alba, A.R., 2007. Solid-phase extraction followed by liquid chromatography-time-of-flight- mass spectrometry to evaluate pharmaceuticals in effluents. A pilot monitoring study. J. Environ. Monit. 9, 719–729.

Gosetti, F., Mazzucco, E., Zampieri, D., Gennaro, M.C., 2010. Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A 1217, 3929–37.

Gracia-Lor, E., Ibáñez, M., Zamora, T., Sancho, J.V., Hernández, F., 2014. Investigation of pharmaceutical metabolites in environmental waters by LC-MS/MS. Environ. Sci. Pollut. Res. Int. 21, 5496–510.

Gracia-Lor, E., Martínez, M., Sancho, J.V.., Peñuela, G., Hernández, F., 2012. Multi-class determination of personal care products and pharmaceuticals in environmental and wastewater samples by ultra-high performance liquid-chromatography-tandem mass spectrometry. Talanta 99, 1011–1023.

Gracia-Lor, E., Sancho, J.V., Hernández, F., 2011. Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1218, 2264–2275.

Grenni, P., Patrolecco, L., Ademollo, N., Tolomei, A., Barra Caracciolo, A., 2013. Degradation of Gemfibrozil and Naproxen in a river water ecosystem. Microchem. J. 107, 158–164.

Gros, M., Petrovic, M., Ginebreda, A., Barceló, D., 2010. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environ. Int. 36, 15–26.

Hernández, F., Grimalt, S., Pozo, Ó.J., Sancho, J.V., 2009. Use of ultra-high-pressure liquid chromatography-quadrupole time-of-flight MS to discover the presence of pesticide metabolites in food samples. J. Sep. Sci. 32, 2245–2261.

Hernández, F., Ibáñez, M., Gracia-Lor, E., Sancho, J.V., 2011. Retrospective LC-QTOF-MS analysis searching for pharmaceutical metabolites in urban wastewater. J. Sep. Sci. 34, 3517–3526.

Hernández, F., Sancho, J.V., Ibáñez, M., Abad, E., Portolés, T., Mattioli, L., 2012. Current use of high-resolution mass spectrometry in the environmental sciences. Anal. Bioanal. Chem. 403, 1251–64.

Hernández, F., Sancho, J.V., Ibáñez, M., Grimalt, S., 2008. Investigation of pesticide metabolites in food and water by LC-TOF-MS. TrAC - Trends Anal. Chem. 27, 862–872.

Hernando, M.D., Gómez, M.J., Agüera, A., Fernández-Alba, A.R., 2007. LC-MS analysis of basic pharmaceuticals (beta-blockers and anti-ulcer agents) in wastewater and surface water. Pharm. Anal. 26, 581–594.

Huet, A.-C., Bienenmann-Ploum, M., Vincent, U., Delahaut, P., 2013. Screening methods and recent developments in the detection of anticoccidials. Anal. Bioanal. Chem. 405, 7733–51.

Ibáñez, M., Gracia-Lor, E., Sancho, J.V., Hernández, F., 2012. 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. J. Mass Spectrom. 47, 1040–6.

Ibáñez, M., Sancho, J.V., Pozo, Ó.J., Niessen, W.M.A., Hernández, F., 2005. Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water. Rapid Commun. Mass Spectrom. 19, 169–178.

IT, 04/2011 Ministerio de Sanidad, subgrupos ATC y principios activos de mayor consumo en el Sistema Nacional de Salud. http://www.msc.es/biblioPublic/publicaciones/recursos_propios/infMedic/porVolumen/home.htm.

Jjemba, P.K., 2006. Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. Ecotoxicol. Environ. Saf. 63, 113–30.

Kaufmann, A., Butcher, P., Maden, K., Walker, S., Widmer, M., 2011. Development of an improved high resolution mass spectrometry based multi-residue method for veterinary drugs in various food matrices. Anal. Chim. Acta 700, 86–94.

Kostopoulou, M., Nikolaou, A., 2008. Analytical problems and the need for sample preparation in the determination of pharmaceuticals and their metabolites in aqueous environmental matrices. TrAC Trends Anal. Chem. 27, 1023–1035.

Lai, F.Y., Ort, C., Gartner, C., Carter, S., Prichard, J., Kirkbride, P., Bruno, R., Hall, W., Eaglesham, G., Mueller, J.F., 2011. Refining the estimation of illicit drug consumptions from wastewater analysis: Co-analysis of prescription pharmaceuticals and uncertainty assessment. Water Res. 45, 4437–4448.

Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S., 2012. Emerging organic contaminants in groundwater: A review of sources, fate and occurrence. Environ. Pollut. 163, 287–303.

Li, W.C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. Environ. Pollut. 187, 193–201.

López-Serna, R., Jurado, A., Vázquez-Suñé, E., Carrera, J., Petrović, M., Barceló, D., 2013. Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain. Environ. Pollut. 174, 305–15.

Marín, J.M., Gracia-Lor, E., Sancho, J.V., López, F.J., Hernández, F., 2009. Application of ultra-high-pressure liquid chromatography-tandem mass spectrometry to the determination of multi-class pesticides in environmental and wastewater samples. Study of matrix effects J. Chromatog. A, 1216, 1410–1420.

Meffe, R., de Bustamante, I., 2014. Emerging organic contaminants in surface water and groundwater: a first overview of the situation in Italy. Sci. Total Environ. 481, 280–95.

Mol, H.G.J., Plaza-Bolaños, P., Zomer, P., De Rijk, T.C., Stolker, A.A.M., Mulder, P.P.J., 2008. Toward a generic extraction method for simultaneous determination of pesticides, mycotoxins, plant toxins, and veterinary drugs in feed and food matrixes. Anal. Chem. 80, 9450–9459.

Mompelat, S., Le Bot, B., Thomas, O., 2009. Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. Environ. Int. 35, 803–14.

Nácher-Mestre, J., Ibáñez, M., Serrano, R., Pérez-Sánchez, J., Hernández, F., 2013. Qualitative screening of undesirable compounds from feeds to fish by liquid chromatography coupled to mass spectrometry. J. Agric. Food Chem. 61, 2077–2087.

Ortiz de García, S., Pinto Pinto, G., García Encina, P., Irusta Mata, R., 2013. Consumption and occurrence of pharmaceutical and personal care products in the aquatic environment in Spain. Sci. Total Environ. 444, 451–465.

Pal, R., Megharaj, M., Kirkbride, K.P., Naidu, R., 2013. Illicit drugs and the environment--a review. Sci. Total Environ. 463-464, 1079–92.

Pérez, S., Barceló, D., 2007. Application of advanced MS techniques to analysis and identification of human and microbial metabolites of pharmaceuticals in the aquatic environment. TrAC Trends Anal. Chem. 26, 494–514.

Postigo, C., López de Alda, M.J., Barceló, D., 2010. Drugs of abuse and their metabolites in the Ebro River basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation. Environ. Int. 36, 75–84.

Reglamento (CEE) n° 2377/90 del Consejo, de 26 de junio de 1990, por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de medicamentos veterinarios en los alimentos de origen animal.

Reglamento (CE) nº 1873/2003 de la Comisión, de 24 de octubre de 2003, por el que se modifica el anexo II del Reglamento (CEE) nº 2377/90 del Consejo por el que se establece un procedimiento comunitario de fijación de los límites máximos de residuos de e medicamentos veterinarios en los alimentos de origen animal.

Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition, Official Journal of the European Union L 268/29; available at http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/EC-1831-2003.pdf. Accessed 25 Apr 2013.

Richardson, S.D., 2008. Environmental mass spectrometry: emerging contaminants and current issues. Anal. Chem. 80, 4373–402.

Richardson, S.D., Ternes, T.A., 2014. Water analysis: emerging contaminants and current issues. Anal. Chem. 86, 2813–48.

Rosal, R., Rodríguez, A., Perdigón-Melón, J.A., Petre, A., García-Calvo, E., Gómez, M.J., Agüera, A., Fernández-Alba, A.R., 2010. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Emerg. Contam. water Occur. fate, Remov. Assess. water cycle (from wastewater to Drink. water) 44, 578–588.

Samra, T., Kuo, J., 2013. Disinfection and Antimicrobial Processes. Water Environ. Res. 85, 1262–1282.

SANCO/12571/2013; European Commission Health & Consumer Protection Directorate-General. Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed. http://www.accredia.it/UploadDocs/4584_AqcGuidance_Sanco_2013_12571.pd.

Scheidweiler, K.B., Desrosiers, N.A., Huestis, M.A., 2012. Simultaneous quantification of free and glucuronidated cannabinoids in human urine by liquid chromatography tandem mass spectrometry. Clin. Chim. Acta. 413, 1839–47.

Seifrtová, M., Nováková, L., Lino, C., Pena, A., Solich, P., 2009. An overview of analytical methodologies for the determination of antibiotics in environmental waters. Anal. Chim. Acta 649, 158–79.

Shah, R.P., Sahu, A., Singh, S., 2010. Identification and characterization of degradation products of irbesartan using LC-MS/TOF, MSn, on-line H/D exchange and LC-NMR. J. Pharm. Biomed. Anal. 51, 1037–1046.

Skopp, G., Pötsch, L., 2004. An Investigation of the Stability of Free and Glucuronidated 11-Nor-Δ9-Tetrahydrocannabinol-9-carboxylic Acid in Authentic Urine Samples. J. Anal. Toxicol. 28, 35–40.

Sousa, M.A., Gonçalves, C., Cunha, E., Hajšlová, J., Alpendurada, M.F., 2011. Cleanup strategies and advantages in the determination of several therapeutic classes of pharmaceuticals in wastewater samples by SPE-LC-MS/MS. Anal. Bioanal. Chem. 399, 807–822.

Stuart, M., Lapworth, D., Crane, E., Hart, A., 2012. Review of risk from potential emerging contaminants in UK groundwater. Sci. Total Environ. 416, 1–21.

Taylor, P.J., 2005. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. Clin. Biochem. 38, 328–34.

Ternes, T., Bonerz, M., Schmidt, T., 2001. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography–electrospray tandem mass spectrometry. J. Chromatogr. A 938, 175–185.

Thomas, K. V, Bijlsma, L., Castiglioni, S., Covaci, A., Emke, E., Grabic, R., Hernández, F., Karolak, S., Kasprzyk-Hordern, B., Lindberg, R.H., Lopez de Alda, M., Meierjohann, A., Ort, C., Pico, Y., Quintana, J.B., Reid, M., Rieckermann, J., Terzic, S., van Nuijs, A.L.N., de Voogt, P., 2012. Comparing illicit drug use in 19 European cities through sewage analysis. Sci. Total Environ. 432, 432–439.

Trufelli, H., Palma, P., Famiglini, G., Cappiello, A., 2011. An overview of matrix effects in liquid chromatography-mass spectrometry. Mass Spectrom. Rev. 30, 491–509.

UNODC 2013 United Nations Office on Drugs and Crime, Vienna. World Drug Report 2013. http://www.unodc.org/unodc/secured/wdr/wdr2013/World_Drug_Report_2013.pdf

UNODC 2006 United Nations Office on Drugs and Crime, Vienna. World Drug Report 2013. https://www.unodc.org/pdf/WDR_2006/wdr2006_volume2.pdf.

Valcárcel, Y., González Alonso, S., Rodríguez-Gil, J.L., Gil, A., Catalá, M., 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84, 1336–1348.

Van Nuijs, A.L.N., Tarcomnicu, I., Simons, W., Bervoets, L., Blust, R., Jorens, P.G., Neels, H., Covaci, A., 2010. Optimization and validation of a hydrophilic interaction liquid chromatography-tandem mass spectrometry method for the determination of 13 top-prescribed pharmaceuticals in influent wastewater. Anal. Bioanal. Chem. 398, 2211–2222.

Verlicchi, P., Galletti, A., Petrovic, M., Barceló, D., Al Aukidy, M., Zambello, E., 2013. Removal of selected pharmaceuticals from domestic wastewater in an

activated sludge system followed by a horizontal subsurface flow bed - Analysis of their respective contributions. Sci. Total Environ. 454-455, 411–425.

Wille, K., De Brabander, H.F., Vanhaecke, L., De Wulf, E., Van Caeter, P., Janssen, C.R., 2012. Coupled chromatographic and mass-spectrometric techniques for the analysis of emerging pollutants in the aquatic environment. Trends Anal. Chem. 35, 87–108.

Wong, C.S., MacLeod, S.L., 2009. JEM spotlight: recent advances in analysis of pharmaceuticals in the aquatic environment. J. Environ. Monit. 11, 923–36.

Yaroshenko, D. V., Kartsova, L.A., 2014. Matrix effect and methods for its elimination in bioanalytical methods using chromatography-mass spectrometry. J. Anal. Chem. 69, 311–317.

CAPÍTULO 8

CONCLUSIONES

Conclusiones

La **conclusión principal** que se deriva de las investigaciones realizadas en la presente Tesis Doctoral es que la cromatografía líquida de ultra-resolución acoplada a la espectrometría de masas presenta un gran potencial tanto para la determinación de residuos contaminantes orgánicos, como para la investigación de metabolitos y productos de transformación en diferentes tipos de muestras. Esta Tesis ha sido desarrollada con el fin de aportar un mayor conocimiento en el campo de la salud pública, particularmente en el ámbito de la contaminación del medio ambiente y de la seguridad alimentaria. Las dos técnicas empleadas en esta Tesis (LRMS con analizador de triple cuadrupolo y HRMS con analizador de tiempo de vuelo) son muy poderosas y complementarias en el campo de trabajo abordado.

Del trabajo realizado en la presente Tesis Doctoral, se pueden extraer las siguientes **conclusiones específicas**:

1. UHPLC-(MS/MS) con triple cuadrupolo es una técnica muy valiosa para la determinación (cuantificación y confirmación) de fármacos, drogas de abuso y sus metabolitos y/o productos de transformación en diferentes tipos de aguas. La excelente sensibilidad y selectividad proporcionadas por esta técnica, junto con su robustez, hacen que sea la herramienta ideal para el desarrollo de métodos multi-residuos de contaminantes emergentes seleccionados, en análisis rápidos y con poca manipulación de muestra.
2. La inyección directa de la muestra de agua en sistemas UHPLC-MS/MS QqQ de última generación ha permitido la detección de numerosos fármacos y drogas de abuso en efluentes urbanos y en aguas superficiales a niveles de sub-ppb sin necesidad de llevar a cabo ningún tratamiento previo de la muestra.

3. El acoplamiento UHPLC-QTOF MS con adquisición en modo MS^E es una herramienta muy poderosa para la elucidación de metabolitos y productos de transformación generados en experiencias de laboratorio en ambientes controlados. Esto es debido a la información adquirida sobre la molécula (des)protonada (función LE) y los iones fragmento (función HE) con medidas de masa exacta.

4. Se ha demostrado la utilidad de las experiencias de degradación (hidrólisis, biodegradación, foto-degradación y/o cloración) para diversos fármacos y drogas de abuso en análisis posteriores realizados en aguas residuales y medioambientales, ya que muchos de los TPs identificados en el laboratorio han sido finalmente detectados en numerosas muestras de agua.

5. La metodología seguida en esta Tesis (experiencias de degradación en muestras de agua fortificadas y posterior investigación de los TPs formados en aguas reales) ha sido aplicada con éxito a diversos fármacos como omeprazol, ibuprofeno, gemfibrozil, irbesartán, ofloxacino y venlafaxina, y drogas de abuso, como el cannabis. Una estrategia análoga ha sido aplicada en experiencias de metabolismo del omeprazol.

6. En ninguna de las aguas analizadas (IWW, EWW y SW) se detectó omeprazol. Sin embargo, se detectaron varios de sus TPs y de sus metabolitos, en algunos casos en todas las muestras de agua, por lo que se concluye que para investigar el impacto del omeprazol en el medio ambiente acuático se debe centrar la búsqueda en metabolitos y TPs relevantes, en lugar del fármaco inalterado.

7. Los resultados obtenidos en la evaluación retrospectiva de los datos, realizada en muestras de agua ya analizadas por QTOF, y la realización de nuevos análisis por QqQ muestran la presencia de diversos metabolitos y TPs (por ejemplo, TPs

de venlafaxina, de ibuprofeno o de gemfibrozil) en aguas, en ocasiones con mayor frecuencia que los fármacos de partida. Por este motivo, se requiere ampliar la investigación sobre estos compuestos, adquiriendo los escasos patrones de referencia disponibles y sintetizando el resto, con el fin de confirmar su identidad de modo inequívoco y posteriormente desarrollar metodología analítica para su adecuada cuantificación en las muestras.

8. La mayoría de los metabolitos y TPs descubiertos en esta Tesis Doctoral son compuestos que, en nuestro conocimiento, no han sido reportados previamente en literatura científica. Por ello, además de su confirmación con patrones de referencia, sería deseable evaluar su potencial toxicidad/peligrosidad en el medio ambiente, al menos para los compuestos más relevantes encontrados en la mayoría de las muestras.

9. Para algunos compuestos de elevado consumo, como irbesartán, venlafaxina, ibuprofeno o gemfibrozil, así como para THC-COOH (principal metabolito de cannabis) se han detectado diversos de sus TPs en las aguas analizadas. Sería recomendable incluirlos en futuros métodos multi-residuo para obtener una visión más realista de los compuestos emergentes que pueden estar presentes en nuestro medioambiente. Para un buen número de fármacos, la inclusión en la metodología analítica únicamente del compuesto inalterado aportará información limitada, pues muchas veces los metabolitos o TPs son más abundantes que el propio fármaco.

10. Se ha desarrollado metodología analítica de *screening* mediante UHPLC-QTOF MS para pienso animal. La validación cualitativa ha permitido establecer los límites de detección de *screening*, que fueron de 0.02 mg/Kg para la mayoría de los 116 fármacos de uso humano y veterinario en las cinco matrices de pienso animal analizadas. La aplicación del método de *screening* a un buen número de

piensos de distintos tipos, y la ampliación de la base de datos hasta más de 600 fármacos, ha permitido la detección de varios compuestos, algunos de los cuales se encuentran actualmente prohibidos, como α y β-nandrolona en pienso bovino, cunícola, caprino y/o porcino. Los resultados preliminares obtenidos en la cuantificación de los compuestos detectados muestran que el QTOF MS podría utilizarse no sólo para el *screening* de un amplio número de fármacos veterinarios, sino también para su adecuada cuantificación. No obstante, se requieren estudios adicionales para corroborar estos resultados.

CHAPTER 8

CONCLUSIONS

Conclusions

The **general conclusion** reached from the research carried out in this Doctoral Thesis is that ultra-high performance liquid chromatography coupled to mass spectrometry (both tandem LRMS and HRMS) is a highly suitable tool for the determination of emerging contaminants, as pharmaceuticals and illicit drugs of abuse, as well as for the investigation of their metabolites and transformation products in different types of samples. This Thesis has been developed as a contribution to increase the knowledge in the public health field, specifically, in environmental pollution and food safety. As demonstrated in this Thesis, the two MS techniques applied (LRMS with triple quadrupole and HRMS using a quadrupole time-of-flight mass analyzer) are very powerful and complementary in this field.

Apart from this general statement, several **specific conclusions** can be extracted:

1. UHPLC-MS/MS with triple quadrupole has been proven to be a highly efficient technique for the determination (quantification and confirmation) of pharmaceuticals, drugs of abuse and their metabolites/transformation products in different types of waters. The excellent sensitivity, selectivity and robustness provided by this technique, make LC-MS/MS the best option for developing rapid multi-residue methods for quantification of selected (target) emerging contaminants, with little sample treatment.
2. Direct injection of the water sample in last-generation UHPLC-MS/MS QqQ systems has allowed the determination of pharmaceuticals and drugs of abuse in effluent wastewaters and surface waters at sub-ppb levels, avoiding any type of sample pre-treatment.

3. UHPLC-QTOF MS acquiring in MS^E is a valuable tool for the identification and elucidation of metabolites and transformation products generated after laboratory controlled conditions. This is possible thanks to the accurate-mass full-spectrum acquisitions provided by QTOF MS at low and high collision energy. Typically, information on accurate mass of the (de)protonated molecule is acquired at the LE function, while fragment ions are commonly present at the HE function.

4. The degradation experiments performed in this Thesis (hydrolysis, biodegradation, photo-degradation and/or chlorination) for selected pharmaceuticals and drugs of abuse have been shown much useful in subsequent analysis carried out in wastewaters and environmental samples, as an important number of TPs identified in laboratory conditions have been finally detected in a notable number of water samples.

5. The methodology followed in this Thesis (degradation experiments in spiked aquatic samples and subsequent investigation of the TPs formed in real-life water samples) has been successfully applied to different pharmaceuticals, such as omeprazole, gemfibrozil, irbesartan, ofloxacin and venlafaxine, and drugs of abuse like cannabis. A similar strategy has been applied to investigate the presence of omeprazole metabolites in water.

6. It is worth noticing that omeprazole was not present in any of the samples analyzed (IWW, EWW and SW) despite its wide human consumption; nevertheless, the presence of several TPs and metabolites was observed in most of samples, even reaching 100% of the samples analyzed for some compounds. It is concluded that the investigation of omeprazole impact on the aquatic environment requires to focus research on its more relevant metabolites and TPs instead of the unchanged compound.

7. Retrospective evaluation of data obtained by QTOF MS in samples previously analyzed, and new analysis performed by LC-MS/MS QqQ, reported the presence of several metabolites and TPs (e.g. from venlafaxine, ibuprofen or gemfibrozil) in waters, in some cases even at higher frequency than the parent pharmaceutical. For this reason, future investigations about the compounds detected would be required. It would be necessary to acquire commercially available reference standards and synthesizing those not available for unequivocal confirmation of their identities and for further development of analytical methodology (based on LC-MS/MS) for correct quantification in samples.

8. From the best of our knowledge, a large number of metabolites and TPs found in this Thesis had not been previously reported in the scientific literature. A further confirmation with reference standards is required in the future, and an evaluation of their potential toxicity/dangerousness in the aquatic environment, at least for the most relevant and frequently detected compounds in water samples.

9. The fact that several TPs from compounds highly consumed, such as irbesartan, venlafaxine, ibuprofen, gemfibrozil, or from the main cannabis metabolite (THC-COOH), have been frequently detected in the samples analyzed, makes recommendable to include them in future multi-residue methods, in order to obtain a more realistic overview of drugs impact on the aquatic environment. For a considerable number of pharmaceuticals, monitoring the unchanged compound would provide only limited information, because metabolites and TPs are often more abundant than the original pharmaceutical.

10. Analytical screening methodology has been developed, based on UHPLC-QTOF MS, for more than 100 human and veterinary pharmaceuticals in five types of

animal feed. Qualitative validation of the screening has allowed establishing the screening detection limits, which were at 0.02 mg/Kg for many analyte/matrix combinations. The application of the screening to a notable number of animal feed, and the extension of a database of more than 600 pharmaceuticals, have allowed detecting several compounds, some of them prohibited, such as α and β-nandrolone, in bovine, rabbit, goat and/or pork feeds. Preliminary data on quantification of the compounds detected in samples show that QTOF MS might be used not only for wide-scope screening of veterinary drugs, but also for their satisfactory quantification. Further research is required to fully support these data.

Sugerencias para trabajos futuros

El trabajo realizado en la presente Tesis Doctoral ha puesto de manifiesto el gran potencial que ofrece la técnica LC-MS, aprovechando la versatilidad de los diferentes analizadores (QqQ y QTOF) para la investigación de fármacos, drogas de abuso y sus metabolitos/TPs tanto en el campo medioambiental como de seguridad alimentaria.

A partir de los resultados obtenidos en esta Tesis, se pueden proponer futuras líneas de trabajo. Algunas sugerencias se describen a continuación:

- Obtener comercialmente o sintetizar los patrones de referencia de los metabolitos y los TPs reportados en esta memoria para la confirmación inequívoca de su identidad.
- Desarrollar, optimizar y validar métodos cuantitativos mediante LC-MS/MS con analizador QqQ para la determinación de los metabolitos y TPs descubiertos en esta Tesis.
- Aplicar la metodología validada a muestras de orina humana para determinar las concentraciones y estimar la excreción del omeprazol y sus metabolitos.
- Aplicar la metodología analítica validada a muestras de agua residual y medioambiental, con el fin de determinar los niveles de metabolitos y TPs en muestras de agua, evaluando de ese modo su impacto ambiental.
- Evaluar la toxicidad de los metabolitos y TPs detectados que no han sido reportados anteriormente.
- Ampliar los estudios sobre fármacos de uso veterinario mediante LC-QTOF MS sobre la base de los resultados preliminares obtenidos en esta Tesis.

Suggestions for future works

The work performed in the present Doctoral Thesis has shown the great potential of LC-MS, taking profit of the versatility of different analyzers (QqQ and QTOF), to investigate pharmaceuticals, drugs of abuse and their metabolites and TPs in the environmental and in food safety fields.

Considering the results obtained in this Thesis, some future works can be proposed:

- To acquire commercially available reference standards of metabolites and TPs reported, and/or synthetize at least the most relevant ones, for the unequivocal confirmation of the compounds tentatively identified.
- To develop, optimize and validate quantitative methods based on LC-MS/MS QqQ to determinate the proposed metabolites and TPs.
- To apply the validated analytical methodology to human urine samples to determine the concentration and estimate the excretion ratios of omeprazole and its metabolites.
- To apply the validated analytical methodology to urban wastewaters and environmental waters, to quantity the concentration levels of metabolites and TPs in water, helping in this way to evaluate their environmental impact.
- To evaluate the toxicity of the detected metabolites and TPs not reported previously through collaborative research with centers specialized in toxicology.
- To advance in the investigation of veterinary drugs in feeds by the use of LC-QTOF MS on the basis of the preliminary results reported in this Thesis

ARTÍCULOS CIENTÍFICOS RELACIONADOS CON LA TESIS

Artículos derivados del presente trabajo

Artículo Científico 1
Fast determination of 40 drugs in water using large volume direct injection liquid chromatography-tandem mass spectrometry
Talanta, 131 (2015) 719-727
Clara Boix, María Ibáñez, Juan V. Sancho, Javier Rambla, José L. Aranda, Salomé Ballester and Félix Hernández

Artículo Científico 2
Investigating the presence of omeprazole in water by liquid chromatography coupled to low and high resolution mass spectrometry: Degradation experiments
Journal of Mass Spectrometry, 48 (2013) 1091-1100
Clara Boix, María Ibáñez, Juan V. Sancho, Wilfried M.A. Niessen and Félix Hernández

Artículo Científico 3
Identification of new omeprazole metabolites in wastewaters and surface waters
Science of the Total Environment, 468 (2014) 706-714
Clara Boix, María Ibáñez, Tatiana Zamora, Juan V. Sancho, Wilfried M.A. Niessen and Félix Hernández

Artículo Científico 4
Investigation of omeprazole and venlafaxine metabolites in wastewater making use of high resolution mass spectrometry
Artículo en proceso
Clara Boix, María Ibáñez, Juan V. Sancho, Renzo Bagnati, Félix Hernández and Sara Castiglioni

Artículo Científico 5
Investigation of cannabis biomarkers and transformation products in waters by liquid chromatography coupled to time-of-flight and triple quadrupole mass spectrometry
Chemosphere, 99 (2014) 64-71
Clara Boix, María Ibáñez, Lubertus Bijlsma, Juan V. Sancho and Félix Hernández

Artículo Científico 6
Biodegradation of pharmaceuticals in surface water and during waste water treatment: identification and occurrence of transformation products
Enviado para su publicación
Clara Boix, María Ibáñez, Juan V. Sancho, John R. Parsons, Pim de Voogt and Félix Hernández

Artículo Científico 7
Qualitative screening of 116 veterinary drugs in feed by liquid chromatography–high resolution mass spectrometry: potential application to quantitative analysis
Analytical Methods, Food Chemistry, 160 (2014) 313-320
Clara Boix, María Ibáñez, Juan V. Sancho, Nuria León, Vicent Yusá and Félix Hernández

Otros artículos relacionados

Artículo Científico 1
Rapid screening of arsenic species in urine from exposed human by inductively coupled plasma mass spectrometry with germanium as internal standard
Journal of Analytical Atomic Spectrometry, 27 (2012) 354–358
Ángel Castillo, **Clara Boix**, Neus Fabregat, Antoni F. Roig-Navarro and Jose A. Rodríguez-Castrillón

Artículo Científico 2
Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment
Science of the Total Environment, 443 (2013) 200-208
Lubertus Bijlsma, **Clara Boix**, Wilfried M.A. Niessen, María Ibáñez, Juan V. Sancho and Félix Hernández

Artículo Científico 3
Improvements in analytical methodology for the determination of frequently consumed illicit drugs in urban wastewater
Analytical and Bioanalytical Chemistry, 406 (2014):4261–4272)
Lubertus Bijlsma, Eduardo Beltrán, **Clara Boix**, Juan V. Sancho and Félix Hernández

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