Análisis y destino de residuos farmacéuticos en aguas subterráneas, superficiales y residuales

Rebeca López Serna

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Programa de Doctorado “Química Analítica del Medi Ambient i la Pol·lució”

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“Si todas las medicinas del mundo fuesen tiradas al mar,
sería lo mejor para la humanidad,
y lo peor para los peces”

Oliver Wendell

ESTA TESIS DOCTORAL SE HA REALIZADO GRACIAS A LA AYUDA FINANCIERA OTORGADA POR EL MINISTERIO DE CIENCIA E INNOVACION MEDIANTE LOS PROYECTOS CEMAGUA CGL2007-64551/HID Y CONSOLIDER-INGENIO 2010 SCARCE CSD2009-00065, Y LA BECA PRE-DOCTORAL PARA LA FORMACIÓN DE PERSONAL INVESTIGADOR (FPI-2008, BES-2008-006719) Y UNA BECA PARA REALIZAR ESTANCIAS BREVES EN EL EXTRANJERO (BES-2008-006719)

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ÍNDICE

1. INTRODUCCIÓN GENERAL

1.1 Fármacos como contaminantes emergentes en el medio ambiente acuático

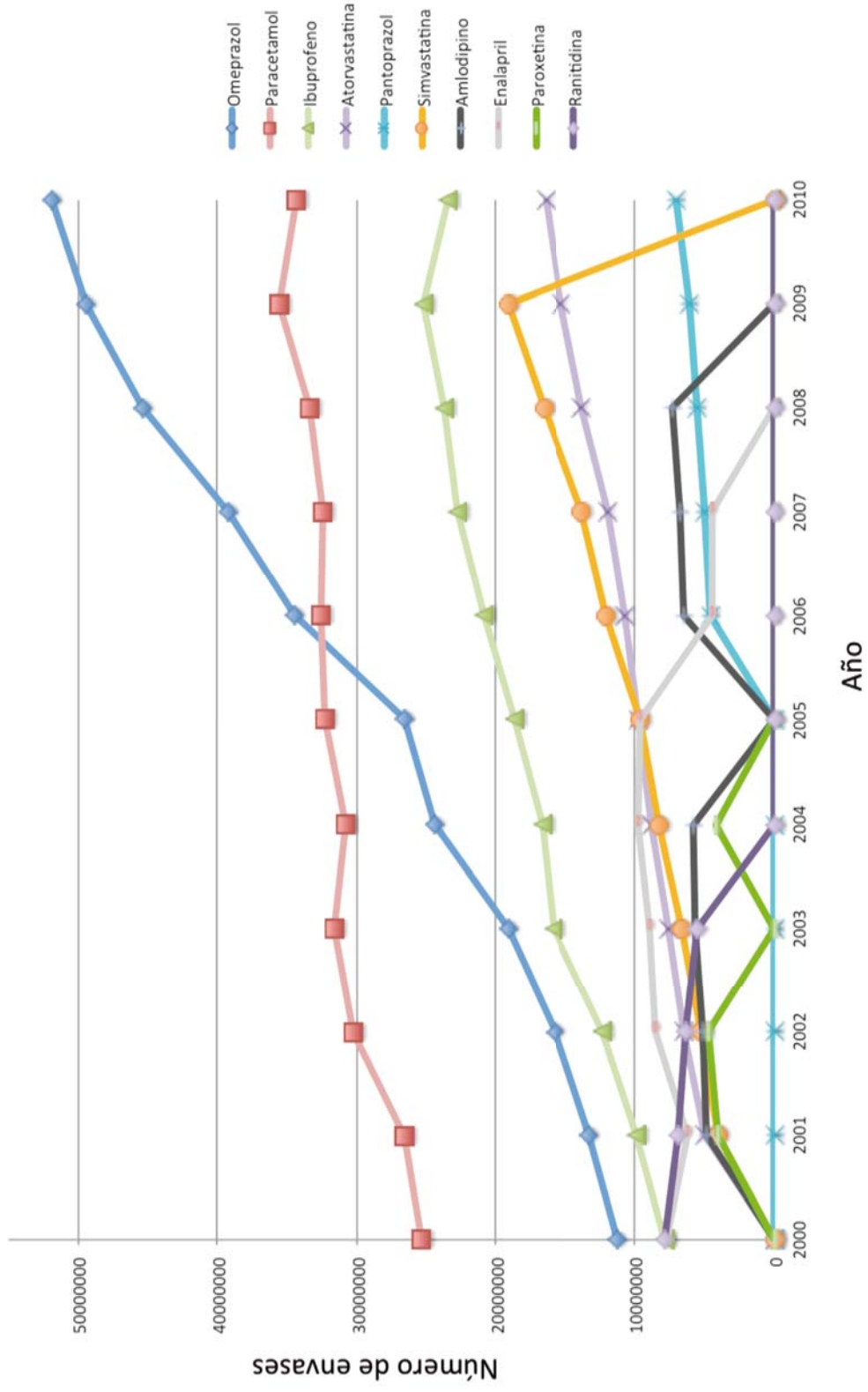
Un fármaco es toda sustancia química utilizada en la prevención, diagnóstico, tratamiento, mitigación y/o cura de una enfermedad. Los fármacos se expenden y utilizan principalmente en la forma de medicamentos, los cuales contienen el o los fármacos, en conjunto con excipientes. Los fármacos pueden ser sintetizados o extraídos de un organismo vivo. En este último caso, debe ser purificado y/o modificado químicamente, antes de ser considerado como tal. La actividad de un fármaco varía debido a la naturaleza de estos, pero siempre está relacionado con la cantidad ingerida o absorbida.

La industria farmacéutica ha sido una de las industrias que ha mostrado un crecimiento más rápido a nivel mundial en los últimos años. Sin embargo, la distribución es inequitativa en el mundo, siendo los países desarrollados los líderes tanto en producción como en consumo de fármacos. En 2010, Norte América (38%), Europa (29%) y Japón (12%) acapararon casi el 79% del mercado global. Por otro lado, regiones en desarrollo, las cuales cuentan con el 85% de la población mundial, contaban con tan solo el 21% del consumo mundial en ese mismo año [1].

Informes de la Agencia Española del Medicamento y Productos Sanitarios sobre el uso de medicamentos extrahospitalarios con receta en España entre los años 1992 y 2006, muestran un aumento en las Dosis Diarias Definidas (DDD) por cada mil habitantes y día, de todas las familias, excepto para los antibióticos que se mantuvieron prácticamente constates entorno a 20 DDD por cada 1.000 habitantes y día, y los antimigrañosos que descendieron de 1.19 a 0.73 DDD por cada 1.000 habitantes y día. El mayor aumento se produjo entre los hipertensivos que pasaron de 81 a 233 DDD por cada 1.000 habitantes y día. Esto se debe, además de al alto consumo individual, como en la furosemida (11 DDD por cada 1.000 habitantes y día en 2006), enalapril (35 DDD por cada 1.000 habitantes y día en 2006) o amlodipino (16 DDD por cada 1.000 habitantes y día en 2006), al gran número de fármacos a la venta pertenecientes a esta familia. Otros fármacos muy consumidos son los antiinflamatorios no esteroides (especialmente debido al ibuprofeno), los ansiolíticos-hipnóticos (especialmente debido al lorazepam, alprazolam y lormetazepam), los antiagregantes (especialmente debido al acido acetilsalicílico), antiasmáticos (fundamentalmente debido al salbutamol), antidiabéticos (fundamentalmente a la metformina), antiulcerosos (fundamentalmente debido al omeoprazol) y hipolipemiantes (especialmente debido a la atorvastatina y simvastina), con DDD por cada 1.000 habitantes y día en 2006 superior a 40 en todos ellos [2].

En la Figura 1 se muestra la evolución en las ventas de 10 de los principios activos más vendidos bajo receta médica durante el periodo 2000-2010 en España.

Figura 1. Evolución del nº de envases vendidos de los 10 principios activos más vendidos bajo receta médica en España durante el periodo 2000-2010



No obstante, hay que tener en cuenta, que los medicamentos expedidos sin receta médica representan también una parte muy importante del consumo.

En cualquier caso, un estudio sobre el consumo de fármacos en España en comparación con el consumo medio en Europa durante las últimas tres décadas [3], ha mostrado que el consumo de fármacos en España, expresado en DDD por cada 1.000 habitantes y día, se encuentra muy cerca del promedio de Europa o incluso por debajo del mismo, para la mayor parte de los grupos terapéuticos, con excepción de los antiulcerosos, ansiolíticos y vasodilatadores periféricos, que superan notablemente el promedio del grupo.

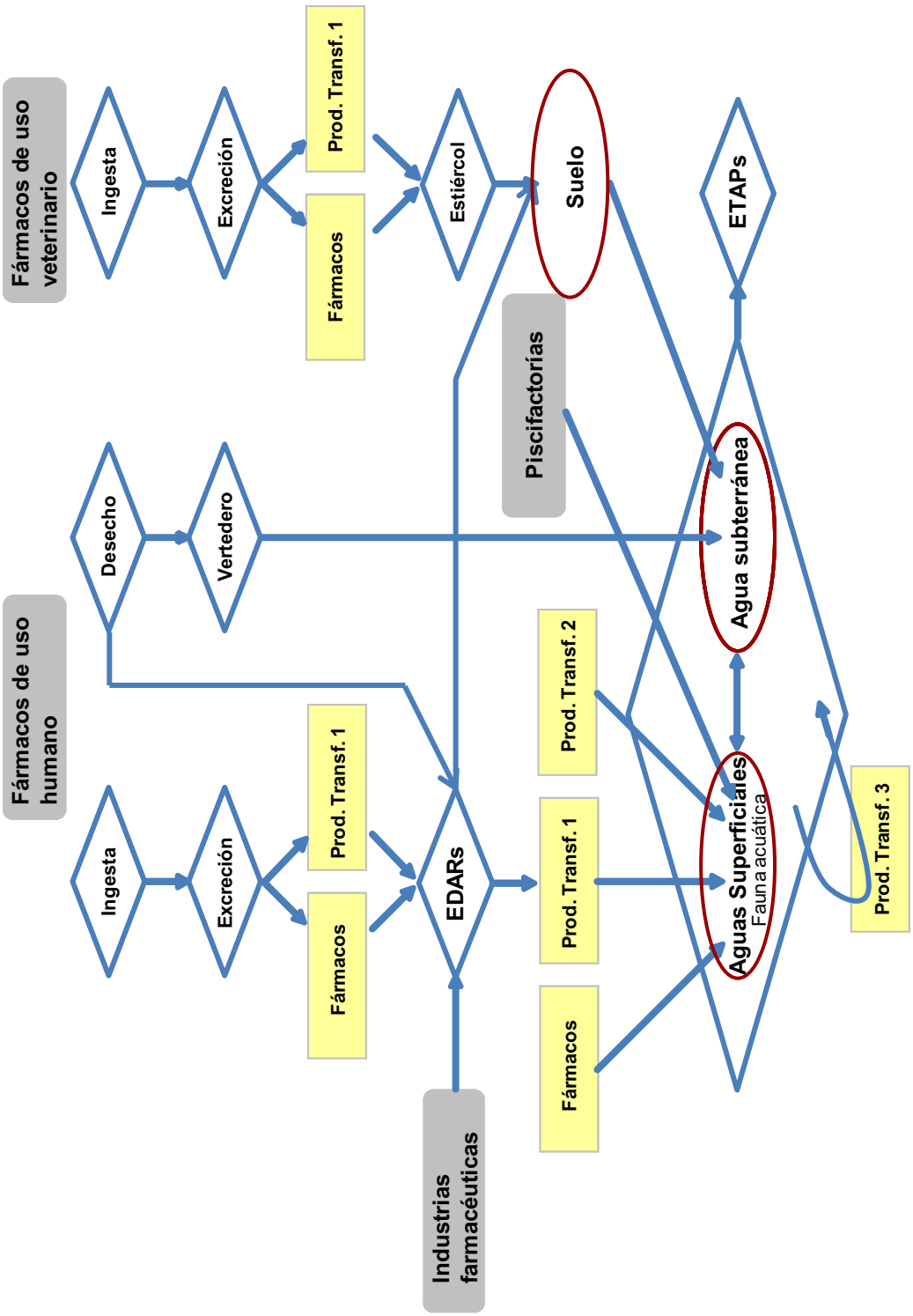
Los fármacos tanto de uso veterinario como humano, y de estos últimos tanto los que se expenden bajo receta como los que no, son encontrados en el medioambiente como contaminantes emergentes.

Los residuos farmacéuticos pueden alcanzar el medioambiente a través de diferentes rutas. Ver Figura 2. Es generalmente asumido que la producción de fármacos en países industrializados está bien controlada y no causa ningún daño al medio ambiente, debido a las restricciones locales normalmente requeridas para permitir la producción. Sin embargo, una fracción substancial de la producción global de fármacos tiene lugar en países donde la producción es de bajo coste como India y China. Informes recientes de la India demuestran que estos sitios pueden emitir cantidades muy elevadas de, por ejemplo, antibióticos, provocando niveles de estas drogas en las aguas superficiales locales mayores que aquellos encontrados en la sangre de los pacientes bajo tratamiento. La principal ruta de los residuos farmacéuticos para alcanzar el medioambiente acuático es probablemente la excreción por parte de los pacientes sometidos a tratamiento farmacológico. Debido a que muchas substancias no son metabolizadas por el cuerpo pueden ser excretadas en forma biológicamente activa, normalmente a través de la orina. Además, muchos substancias farmacéuticas no son completamente adsorbidas por el intestino (cuando la via de administración es oral) hacia el torrente sanguíneo, permaneciendo en el intestino y finalmente son excretados a través de las heces. De ahí que, tanto la orina como las heces de pacientes tratados contengan residuos farmacéuticos. Una fuente adicional de contaminación medioambiental con fármacos es un inadecuado desecho (a través del lavabo, retrete y/o la basura) de medicinas sobrantes o caducadas, así como de sus envases [4]. En los países europeos existen normalmente sistemas de retirada de este tipo de fármacos, aunque no siempre se hace uso de ellos. Mientras que por ejemplo en los Estados Unidos (EEUU), solo existen iniciativas voluntarias a nivel local. En el caso de España la asociación sin ánimo de lucro SIGRE, coloca puntos de recogida en las farmacias. Y es que, en los últimos años, ha ganando fuerza la idea de que las compañías farmacéuticas deben responsabilizarse de la vida completa de sus productos, desde su producción hasta su eliminación [5]. Una adecuada destrucción de los residuos farmacológicos debería llevar a productos sin ninguna actividad farmacéutica ni ecotoxicológica. Además, los residuos no deberían actuar como componentes en la formación medioambiental de nuevos productos.

Se considera que la incineración a alta temperatura (>1.000 grados Celsius) cumple con ambos requerimientos, pero incluso así las cenizas generadas deben ser tratadas adecuadamente. Resultados de un estudio [6] mostró que a la hora de deshacerse de un medicamento, el 63.2% de los participantes los tira al cubo de la basura, el 21.8% los devuelve a la farmacia, el 11.5% los echa por el lavabo o retrete, y el 3.5% restante los guarda en casa. Solo la mitad de los encuestados son conscientes de que los fármacos podrían ser potencialmente dañinos para el medioambiente. Tras la examinación de factores relevantes de percepción de riesgo, no se encontró una conexión definitiva entre percepción y educación o ingresos económicos [6]. Otros orígenes lo constituyen el uso de lodos de depuradora usados como fertilizantes en la agricultura y el riego con agua residual tratada, así como fugas en la red de alcantarillado [7]. Los fármacos usados en medicina veterinaria, o como aditivos en la alimentación animal, son excretados al suelo directamente, o el estiércol es utilizado como fertilizante, en muchos casos. Si las sustancias son hidrófobas, pueden unirse fuertemente a las partículas del suelo, presentando poca tendencia a filtrarse hacia el agua subterránea o aguas superficiales cercanas. En ese caso, si se trata de sustancias con poca degradabilidad biológica, pueden pasar a la cadena alimentaria. Pero la mayoría de los residuos son solubles en agua y, por lo tanto, pueden ser lixiviados con la lluvia o el deshielo de la nieve y alcanzar tanto las aguas subterráneas como los torrentes de agua superficial. Así, debido a su general no volatilidad y solubilidad en agua, a menudo es el agua el compartimento ambiental de destino de estos contaminantes.

Por lo tanto, la mayor parte de los fármacos que se encuentran en el medioambiente han pasado previamente por las Estaciones Depuradoras de Aguas Residuales (EDARs) [8]. Como se explica más adelante en la sección 1.2.1 de esta introducción, las EDARs cuentan con una serie de tratamientos físicos, químicos y biológicos, que disminuyen, en gran medida, la carga contaminante de las aguas residuales. No obstante, no están especialmente diseñadas para la eliminación de este tipo de contaminantes. Durante uno de los tratamientos habituales, el tratamiento secundario, se puede llegar a degradar el contenido orgánico de las aguas residuales en más de un 90%. No obstante, el comportamiento de los fármacos durante este tratamiento es impredecible. Puede ser muy eficaz para algunos de ellos, y transparente para otros, habiendo un gran desconocimiento acerca de su causa. En la actualidad, y cada vez más, las EDARs cuentan, también, con técnicas avanzadas como la irradiación UV o la ozonificación, que degradan la materia orgánica no eliminada en el tratamiento biológico. Un tratamiento óptimo con estos métodos, puede llegar a destruir hasta el 80% o más, de los residuos farmacológicos en el agua. Un paso final con carbón activo o filtración por membrana, puede eliminar posibles productos de degradación reactivos procedentes del tratamiento por UV u ozono. En cualquier caso, para los residuos excretados por el organismo en forma de conjugado, es decir, unidos a un ácido biliar, una desconjugación puede tener lugar durante el tratamiento en las EDARs, provocando la paradoja de niveles de fármaco libre más altos a la salida que a la entrada de la planta de tratamiento.

Figura 2. Origen y destino de los fármacos y sus productos de transformación en el medioambiente.



Los fármacos han sido ampliamente ignorados durante años debido a su relativamente baja presencia en las aguas superficiales comparada con la de los contaminantes convencionales [9]. A pesar, de que ya en los años '60, ecologistas y toxicólogos empezaron a expresar preocupación sobre la presencia de fármacos en el agua de bebida, no fue hasta una década más tarde cuando fue por primera vez documentada. Así, en 1975 y 1977, estudios encontraron los ácidos clofíbrico y salicílico a nivel traza en aguas tratadas [10]. Pero, no obstante, no fue hasta los años '90, que la contaminación del agua por fármacos empezó a ser un tema de preocupación medioambiental [7]. Esto conllevó la aparición de estudios de presencia de fármacos en diferentes compartimentos ambientales en todo el mundo. Así, un estudio del 2002 de la USGS (*United States Geological Survey*) [11] publicó cantidades detectables de fármacos y otros productos de cuidado personal en el 80% de las muestras en 139 cauces de agua en los EEUU. Los fármacos más comúnmente detectados fueron fármacos sin receta, hormonas y antibióticos. Otro estudio posterior en el 2006 también de la USGS encontró concentraciones detectables de 28 fármacos en efluentes de EDARs, aguas subterráneas y sedimentos. Las clases terapéuticas incluían antibióticos, analgésicos y anti-inflamatorios, reguladores de lípidos, betabloqueantes, antiepilépticos y hormonas esteroides.

La presente tesis presenta varios estudios de presencia de fármacos en aguas residuales de EDARs españolas, en varios ríos de la Península Ibérica, aguas subterráneas urbanas bajo la ciudad de Barcelona (España), y agua potable de una planta depuradora del noreste de la Península Ibérica. Los resultados obtenidos están ampliamente discutidos en el capítulo 3 de la presente tesis.

Como para los demás contaminantes emergentes, el conocimiento de los efectos ecotoxicológicos de los fármacos (toxicidad individual, toxicidad de mezclas, toxicidad aguda, toxicidad crónica, disrupción endocrina, bioacumulación, magnificación) sobre los seres vivos es limitado [12]. Los fármacos, una vez en el medio ambiente, pueden entrar en contacto con organismos vivos que desarrollan su vida en los medios contaminados. Hay que tener en cuenta, que aunque encontrados a nivel traza (ejemplo, en las aguas medioambientales, del orden de ng L^{-1} – µg L^{-1}), los fármacos son compuestos con actividad inherente a bajas concentraciones, son diseñados para interactuar con procesos biológicos [13], lo que les confiere una toxicidad persé. Estos organismos podrían, por lo tanto, verse afectados por interacción directa con ellos. Por otro lado, en función de su naturaleza, también puede ocurrir que se incorporen a la cadena trófica y que la bioacumulación se magnifique a medida que se asciende en ella. Este fenómeno puede terminar causando efectos a corto o largo plazo en eslabones de dicha cadena alimentaria, aunque no estén en contacto directo con el compartimento ambiental contaminado. Debido a su elevada solubilidad y volatilidad, es el medio acuático el receptor de estos contaminantes por excelencia, así se han encontrado fármacos, en las aguas medioambientales de todo el mundo. Los organismos acuáticos son, por lo tanto, especialmente vulnerables a sus efectos. Investigadores de la Universidad del Estado de Washington han encontrado en

ranas una clase de antidepresivos lo que puede estar ralentizando significativamente su desarrollo. La cada vez más elevada presencia de estrógenos y otras hormonas en las aguas residuales debido a las píldoras anticonceptivas y las terapias hormonales, se ha relacionado con el aumento en la feminización de peces y otros organismos acuáticos expuestos [14]. En general, los fármacos pueden producir tanto feminización como masculinización de diferentes peces, impactando, por lo tanto, en sus niveles de reproducción [5]. Otro estudio de la Universidad de Umea [15] expuso a la trucha arcoíris a agua residual tratada no diluida en tres diferentes lugares en Suecia. La exposición se prolongó durante 14 días a lo largo de los cuales 25 fármacos fueron medidos en el plasma sanguíneo. La hormona levonorgestrel fue detectada en el plasma sanguíneo de los peces entre 8.5 y 12 ng mL^{-1}, lo cual excedía el nivel terapéutico en plasma humano. Los estudios muestran que los niveles de levonorgestrel medidos en el efluente en las tres áreas reducían la fertilidad de dicha trucha. Esta misma universidad acaba de publicar datos de ecotoxicidad de la droga psiquiátrica oxacepam [16]. Aseguran que las concentraciones de este fármaco encontradas habitualmente en las aguas superficiales influenciadas por efluentes de EDAR, son capaces de alterar el comportamiento y el régimen de alimentación de la perca europea (*Perca fluviatilis*). Así, individuos expuestos a agua con concentraciones de 1.8 µg L^{-1} exhibieron una mayor actividad, menor sociabilidad y una frecuencia de alimentación más alta, lo que tiene consecuencias ecológicas y evolucionarias. También sobre la trucha arcoíris hay estudios de impacto de exposiciones ambientales de diclofenac [17]. El diclofenac es una antireumático y antiflogístico [18]. En las aguas superficiales, las concentraciones máximas pueden llegar a 2 g L^{-1} [19]. [20] determinó que la trucha arcoíris expuesta a diclofenaco durante cuatro semanas desarrollaba diferentes alteraciones tanto en los riñones como en la agallas a partir de la concentración umbral de 5 g L^{-1}. Algunos investigadores sugieren que concentraciones de etinilestradiol, un estrógeno usado en los anticonceptivos orales y uno de los fármacos más comúnmente recetados, pueden causar disrupción endocrina en la vida acuática y anfibios a concentraciones tan bajas como 1 ng L^{-1} [21]. Los organismos no acuáticos, no obstante, también necesitan en mayor o menor medida agua para vivir. Así por ejemplo, los humanos recurren a las aguas medioambientales para obtener agua de bebida. Hay varios estudios que muestran que incluso tratada, el agua potable puede contener trazas de estos compuestos puesto que no existe regulación al respecto [10, 22]. En cualquier caso, a día de hoy ningún estudio ha mostrado un impacto directo sobre su salud [23]. Sin embargo, la ausencia de datos empíricos no debe descartar la posibilidad de resultados adversos debido a interacciones o exposiciones de larga duración a estas sustancias. Se hace necesario, por lo tanto, más investigación para determinar los efectos de los humanos en exposiciones durante periodos prolongados a bajos niveles de fármacos, así como a mezclas de éstos [24]. Además, muchos investigadores han especulado sobre el potencial para inducir resistencia antibiótica. Un estudio encontró 10 antibióticos diferentes en efluentes de EDARs, aguas superficiales y sedimentos [12]. Algunos microbiólogos creen que si las concentraciones de antibiótico son mayores que la concentración mínima inhibitoria (*Minimum Inhibitory Concentration*, MIC) de una especie de bacteria, se ejerce una presión

selectiva que favorece a las bacterias más resistentes. Por ejemplo, el MIC de la eritromicina, que es efectiva contra el 90% de las Campylobacter (bacterias crecidas en el laboratorio, y patógeno alimentario más común en los EEUU), es 0.06 ng mL^{-1} [25]. Un estudio encontró que la concentración media de eritromicina, antibiótico comúnmente prescrito, era 90 ng L^{-1} en efluentes de EDARs [12], justo por encima del MIC para el Campylobacter. En 2006 [26] sugirió la introducción de un nuevo concepto, la ecofarmacovigilancia [27]. Ésta es una rama de la farmacología y una forma de fármacovigilancia [28] que estudia la entrada de productos farmacéuticos en el medioambiente tras su uso en humanos o animales, y su impacto sobre los ecosistemas [29-31]. Se encarga de la detección, evaluación, entendimiento y prevención de efectos adversos de fármacos en el medioambiente. La ecofarmacología, en cambio, es un concepto más amplio, que estudia la entrada de fármacos en el medioambiente a través de cualquier ruta y a cualquier concentración [29].

1.1.1 Fármacos quirales

Una molécula quiral es aquélla que no es superponible con su imagen especular. Esto, en los compuestos orgánicos, normalmente ocurre cuando en la molécula hay al menos un átomo de C, P, S o N, con cuatro sustituyentes distintos formando un tetraedro. Las dos imágenes especulares de una molécula son llamados isómeros enantioméricos. Existen reglas para clasificarlos estructuralmente como D o L, y R o S.

Normalmente ambos presentan las mismas propiedades físico-químicas. Una excepción es que giran el plano de la luz polarizada los mismos grados, pero en sentidos contrarios, uno hacia la derecha (denominado dextrógiro, d o (+)), y otro hacia la izquierda (denominado levógiro, l o (-)). En el racemato, es decir, la mezcla equimolar de ambos enantiómeros, por lo tanto, ambos efectos se compensan, y la luz polarizada no gira a su través. Sin embargo, los isómeros enantioméricos se comportan de manera diferente cuando interaccionan con sistemas biológicos. Por lo tanto, la quiralidad juega un papel importante en la vida de los seres vivos. Muchas moléculas biológicamente activas son quirales, como por ejemplo, los aminoácidos (que posteriormente dan lugar a las proteínas) y los glúcidos. En los organismos vivos, éstos normalmente presentan una quiralidad común. Así, todos los aminoácidos naturales son L y todos los glúcidos D. Los encimas, que son proteínas, son quirales, y a menudo distinguen entre los dos enantiómeros de un substrato quiral. Si imaginamos una encima como una cavidad con forma de guante, si este guante corresponde a la mano derecha, uno de los enantiómeros sustrato encajaría y se uniría, mientras que el otro encajaría mal y probablemente no se uniría.

Más de la mitad de las drogas que actualmente están en uso son quirales. Cabe esperar que los enantiómeros de una misma droga tengan propiedades fisicoquímicas similares pero

difieran en sus propiedades biológicas [32]. Así, los procesos de distribución, metabolismo y excreción, normalmente favorecen uno de los enantiómeros frente al otro. Esto resulta del hecho de que los enantiómeros reaccionan estereoselectivamente en sistemas biológicos, por ejemplo con las encimas. Además, puesto que presentan diferentes actividades farmacológicas, los compuestos quirales pueden diferir también en toxicidad. La talidomida representa un buen ejemplo. Como resultado de la administración de la forma racémica de este sedante a mujeres embarazadas, miles de niños nacieron con deformaciones en los años 60. La forma dextrógira de la talidomida es la terapéutica (tiene propiedades tranquilizantes) y sin efectos secundarios. La forma levógira, sin embargo, es la teratogénica, provocando las malformaciones de los embriones cuando se administra a las mujeres embarazadas. No obstante, una vez en el cuerpo humano la primera se interconvierte en vivo al enantiómero tóxico. Por lo tanto, incluso tras la administración exclusiva del enantiómero bueno, pueden todavía darse efectos negativos.

Los metabolitos de fármacos quirales, pueden ser también quirales. Pero también se pueden obtener metabolitos quirales de fármacos no quirales (por ejemplo, el albendazol y la risperidona son compuestos no quirales que son transformados en metabolitos quirales).

Desafortunadamente, muchas drogas quirales se sintetizan aún hoy como racematos. Esto puede deberse a que su separación quiral es difícil, o el coste de su síntesis estereoselectiva es demasiado alto, o simplemente porque cuando la droga se desarrolló por primera vez, los ensayos clínicos de farmacodinámica, farmacocinética, toxicología y teratología, se llevaron a cabo solo con la mezcla racémica [33, 34].

Hasta la fecha, los estudios de contaminación medioambiental por fármacos, han considerado generalmente los enantiómeros de los compuestos quirales conjuntamente. Sin embargo, debido a su diferente interacción con los sistemas biológicos, éstos pueden presentar destinos y concentraciones dispares en los compartimentos medioambientales. Además, cada isómero quiral puede tener asociada una ecotoxicidad distinta. Así, por ejemplo, el S-propranolol presenta mayor toxicidad crónica en ciertos organismos acuáticos. [35]. Por todo esto, en la actualidad comienzan a aparecer estudios que consideran los enantiómeros de los fármacos quirales como residuos diferentes.

En el capítulo 2 de la presente tesis se incluye un trabajo de determinación multi-residuo enantiomérico de varios fármacos quirales en aguas superficiales de la cuenca del río Guadalquivir (Sur de España), y en aguas residuales de entrada y salida a las principales EDARs asociadas.

1.1.2 Productos de transformación

Producto de transformación de un fármaco, es un término muy amplio que incluye todo subproducto generado a partir de un fármaco, ya sea tras un proceso biológico, químico o físico. Los productos de transformación generados tras la metabolización del fármaco en el organismo receptor, son además denominados metabolitos.

No obstante, la clasificación de los productos de transformación de un fármaco en metabolitos y otros subproductos no es siempre clara. En algunos casos, las reacciones del metabolismo humano pueden estar muy cerca de las de biodegradación en las plantas de tratamiento o en el medioambiente. También puede darse el caso de que fármacos diferentes generen los mismos productos de transformación. Así por ejemplo, la acetoaminoantipirina y la formilaminoantipirina son metabolitos del metamizol (un analgésico no esteroide) así como subproductos microbianos de la dimetilaminofenazona [36, 37]. Otro ejemplo, el hidroxil-ibuprofeno y el carboxi-ibuprofeno son subproductos del metabolismo humano del ibuprofeno, pero también de biodegradación en fagos activos de las EDARs [38].

Por degradación de un fármacos se entiende eliminación biológica, química o física de éste, sin tener en cuenta si el fármaco se ha mineralizado o transformado en subproductos [39].

Una vez en el cuerpo humano/animal, la molécula original de la sustancia farmacéutica (es decir, la molécula administrada) es sometida a una serie de reacciones bioquímicas. Por ejemplo, la eliminación de los fármacos en la orina o heces es gobernada por el metabolismo hepático [38, 40, 41]. Éste consiste en un primer paso que incluye oxidación, reducción o rotura hidrolítica del fármaco, conduciendo a moléculas más polares que los compuestos padre. Y un segundo paso de conjugación, consistente en la transferencia de un grupo polar al compuesto padre o metabolitos, como por ejemplo glucuronización o sulfatación. Con ello, los metabolitos resultantes son los suficientemente hidrofílicos e hidrosolubles como para ser eliminados a través de la orina y/o las heces. Otra característica común de la metabolización es la inducción a la pérdida de actividad farmacéutica del fármaco nativo. No obstante, esto no siempre es así. De hecho, por ejemplo las pro-drogas, por definición, son activas solo tras la activación metabólica mediante los sistemas encimáticos de los compuestos padre al/los metabolito/s. Son pro-drogas los fibratos como el fenofibrato o el clofibrato; los antineoplásticos como la ciclofosfamida, ifosfamida [42], 5-fluorouracil, etc. [36].

De acuerdo con las directrices de la European Medicines Agency (EMEA) en Europa y la Food and Drug Administration (FDA) en los EEUU, los nombres y fórmulas de los metabolitos y sus porcentajes de excreción deberían estar en los archivos de autorización de venta de los correspondientes fármacos. También se pueden encontrar datos interesantes para algunas sustancias en la base de datos electrónica Martindale.

Los fármacos, pueden ser clasificados en cuatro clases según su proporción de excreción bajo su forma original [43], es decir, respectivamente, excreción baja (≤ 5%), como es el caso, por ejemplo, del acetaminofén, la carbamacepina, el diacepam y la atorvastatina; excreción moderadamente baja (6-39%), donde se incluyen, por ejemplo, el tramadol, la simvastatina, la fenobarbitona y el metronidazol; excreción relativamente alta (40-69%), como por ejemplo, la atropina, la ampicilina, la didanosina y el trimetoprim; y excreción alta (≥70%), como la observada, por ejemplo, para el baclofen, la gabapentina, el genaconazol y la tetraciclina. La Tabla 1 muestra los porcentajes de excreción de la forma administrada de una selección de fármacos tras su uso terapéutico.

Tabla 1. Porcentajes de excreción de la forma administrada de una selección de fármacos tras su uso terapéutico

Grupo terapéutico	Fármaco	Porcentaje de excreción (%)	Referencia
Analgésicos y anti-inflamatorios	Ácido acetilsalicílico	2-30	[43]
	Diclofenaco	15	[36, 43]
	Ibuprofeno	1-10	[36, 43]
	Morfina	71.6	[36, 43]
	Acetaminofén	<5	[36, 43]
	Codeina	70	[36]
	Tramadol	15-35	[36]
	Ácido 5-aminosalicílico	<12	[36]
Reguladores de lípidos	Bezafibrato	45-50	[36, 43]
	Simvastatina	10-15	[36, 43]
Drogas de uso psiquiátrico	Atropina	50	[43]
	Baclofen	70-85	[43]
	Carbamacepina	1-2	[36, 43]
	Diacepam	1	[36, 43]
	Fluoxetina	2.5-11	[43]
	Naltrexona	<1	[43]
	Fenobarbitona	25	[43]
	Primidona	15-40	[36, 43]
	Diltiacem	2-4	[36]
	Gabapentina	100	[36]
Antihistamínicos	Domperidona	10	[43]
	Ranitidina	30-70	[36, 43]
	Cimetidina	48-75	[36]
Antibióticos	Aciclovir	0,42	[43]
	Amoxicilina	60-90	[36, 43]
	Ampicilina	30-60	[43]
	Cloramfenicol	5-12	[36, 43]
	Ciprofloxacina	50-84	[36, 43]
	Clindamicina	14	[43]

Antibióticos	Dicloxacilina	50	[43]
	Doxiciclina	70	[36, 43]
	Etambutol	50-80	[43]
	Cefalexina	80	[43]
	Didanosina	50	[43]
	Fluconazol	62-80	[43]
	Flucitosina	90	[43]
	Genaconazol	76-87	[43]
	Metronidazol	20-40	[36, 43]
	Minociclina	60	[43]
	Norfloxacin30		[43]
	Sulfametoxazol	15-30	[36, 43]
	Tetracilina	80-90	[43]
	Trimetroprim	60	[43]
	Valaciclovir	57-65	[43]
	Eritromicina	5-10	[36]
	Norfloxacina	30	[36]
	Espiramicina	20	[36]
	Claritromicina	25	[36]
	Tetraciclina	80-90	[36]
	Trimetoprim	60-80	[36]
	Ceftriaxona	70	[36]
Drogas cardiovasculares	Atorvastatin	5	[43]
	Clonidina	40-60	[43]
	Digoxina	50-70	[36, 43]
	Enalapril	30-36	[36, 43]
	Furosemida	40-90	[36, 43]
	Hidroclorotiacida	24	[43]
	Labetalol	1.61	[43]
	Procainamida	30-70	[43]
	Quinapril	2.6	[43]
	Quinidina	20-50	[43]
	Ramipril	25.2	[43]
	Verapamil	<4	[43]
	Propranolol	<0.5	[36]
	Metoprolol	3-30	[36]
	Acebutolol	39	[36]
	Atenolol	50-90	[36]
	Sotalol	>75	[36]
	Valsartan	80	[36]
β-agonistas	Salbutamol	30	[36]
Antineoplásticos	Idarubicino	5	[43]
	Ciclofosfamida	5-20	[36]
	Ifosfamida	<15	[36]
	5-fluorouracilo	11-20	[36]

Observando la tabla anterior, aunque por poco, la mayoría de los fármacos corresponden a la clase de excreción moderadamente baja (es decir, entre 6 y 39%). Los restantes, están homogéneamente distribuidos en las otras tres clases. Así, si tomásemos como referencia la situación descrita por esta tabla, parece legítimo sospechar la presencia de metabolitos en los compartimentos acuáticos tras su vertido en las aguas residuales.

En [36, 43], están publicados también los metabolitos excretados y sus porcentajes de excreción durante el uso terapéutico de un gran número de fármacos de uso humano y veterinario. Es interesante apuntar que entre los numerosos metabolitos que pueden formarse durante la metabolización, normalmente solo un pequeño número de ellos es excretado. Por ejemplo, la carbamacepina puede transformarse en 33 metabolitos [44], pero solo dos de ellos son masivamente excretados, la 10,11-dihidro-10-11-dihidroxicarbamacepina y la 10,11-dihidro-10-11-epoxicarbamacepina.

Una vez excretados en las aguas residuales, los fármacos y sus metabolitos llegan a las aguas ambientales o a las EDARs (Figura 2). En ambos casos, pueden sufrir subsecuentes transformaciones. Nuevos productos de biotransformación pueden formarse a partir de fármacos o sus metabolitos tras el tratamiento secundario llevado a cabo por bacterias en las EDARs. Si posteriormente, una etapa de desinfección es llevada a cabo mediante cloro, ozono (procesos químicos) o radiación UV (proceso físico), nuevos productos pueden formarse. Finalizado el tratamiento, el efluente de la EDAR es vertido a las aguas medioambientales. Ahí, este posible cóctel de fármacos y derivados, pueden ser objeto de procesos biológicos o químicos, mediante interacción con microorganismos o reactivos naturalmente presentes en la naturaleza, así como transformaciones fotoquímicas por parte de la radiación solar. Estas aguas ambientales son utilizadas como fuente de agua potable. Para ello, son sometidas a procesos de desinfección (cloro, ozono, UV), y por lo tanto, nuevos productos de transformación pueden formarse, estando los humanos expuestos directamente a ellos.

En general, se desconocen los productos de transformación producidos en cada caso, y su identificación supone una tarea ardúa y laboriosa, que se puede expresar coloquialmente como “buscar una aguja en un pajar”. No obstante, incluso de los conocidos existen muy pocos estudios de presencia en aguas medioambientales en todo el mundo [45-52]. Los tres últimos trabajos analizan también aguas potables. Algunos de los estudios anteriores, [53-55] y [56] analizaron productos de transformación en aguas residuales. El capítulo 3 de la presente tesis discute la presencia de un elevado número de productos de transformación, muchos de ellos con conocida actividad farmacéutica remanente, junto con los fármacos padre en aguas superficiales de la cuenca del río Ebro, al noreste de la península ibérica, así como en aguas subterráneas urbanas bajo la ciudad de Barcelona (España).

Los productos de transformación formados, pueden presentar una toxicidad y persistencia impredecible, que podrían ser incluso superiores a la de los fármacos padre. Muchos

metabolitos, mantienen incluso actividad farmacéutica del padre. Por lo tanto, los productos de transformación deberían ser tenidos en cuenta en la valoración del riesgo ambiental de los compuestos padre, máxime sabiendo que muchos fármacos no se excretan en su forma original, sino que se metabolizan extensamente [57].

Como cabe esperar, hay muy pocos informes especializados disponibles en relación a los efectos tóxicos potenciales de estos compuestos. En ellos se han llevado a cabo bioensayos sobre bacterias, algas, rotíferos, y microcrustáceos para valorar toxicidad aguda y crónica. También se han usado SOS chromotests y fluctuación Ames para detectar posible genotoxicidad de algunos fotoderivados [39]. Los resultados obtenidos hasta ahora de la valoración de la ecotoxicidad de los fotoproductos del diclofenaco, naproxeno y los fibratos, por ejemplo, constituyen pruebas bien establecidas de que pueden presentar toxicidad aguda y crónica mayores que los compuestos padres, y no se pueden excluir la genotoxicidad y efectos mutagénicos [58].

1.2 Tratamiento de depuración de aguas

1.2.1 Tratamiento de aguas residuales

Las aguas residuales contienen desechos urbanos, agrícolas, o bien proceder de los variados procesos industriales. En muchos países en vías de desarrollo, la mayor parte de las aguas residuales domésticas e industriales se descargan sin ningún tratamiento previo o tras solo tratamiento primario. En Latinoamérica, solo en torno al 15% de las aguas residuales recogidas pasan a través de plantas de tratamiento (con variables niveles de tratamiento real). En Venezuela, el 97% del agua residual del país es descargada sin trato alguno en el medio ambiente [59]. La mayor parte del África subsahariano y la India, carecen de sistemas de tratamiento de aguas residuales. Por otro lado, en Israel, alrededor del 50% del agua usada en agricultura (1 billón de metros cúbicos en 2008) consiste en agua residual reutilizada. Singapur, es el país del mundo con mayor reutilización, y así tiene incluso implementada tecnología para producir agua potable a partir de agua residual [60].

En Norteamérica y la Unión Europea, es común el uso de EDARs municipales que recogen, mediante sistemas de alcantarillado, las aguas residuales producidas en los núcleos urbanos. Incluyen, por lo tanto, aguas residuales domésticas, así como procedente de comercios e industrias, siempre que las características de éstas sean compatibles con el funcionamiento de la planta, y ésta sea capaz de reducir su contaminación a niveles aceptables de vertido. En caso contrario, las industrias en cuestión deberán tratar previamente sus aguas antes de verterlas en el sistema de alcantarillado municipal o incluso hacerse cargo de su total tratamiento. En algunos casos, también recogen el agua de lluvia. En núcleos de población pequeños se recurre, en muchos casos, a fosas sépticas cuyo contenido se traslada periódicamente a la correspondiente EDAR más cercana.

Típicamente, el tratamiento en las EDARs consta de tres etapas denominadas tratamiento primario, secundario y terciario, respectivamente. No obstante, suelen estar precedidas de una etapa de pretratamiento, consistente en desbaste, desarenamiento, ecualización de flujo y eliminación de grasas.

El tratamiento primario consiste en mantener temporalmente el agua residual quieta en un depósito donde los sólidos más pesados puedan depositarse en el fondo (proceso denominado sedimentación primaria) y los aceites, grasas y otros sólidos ligeros floten en la superficie. Se retiran, entonces, los materiales depositados o flotantes y el líquido resultante pasa al tratamiento secundario.

El tratamiento secundario consiste en la conversión progresiva de la materia orgánica disuelta usando bacterias adecuadas, generalmente presentes en las propias aguas. Una de las técnicas más comunes es la de fangos activos (CAS, *Conventional Activated Sludge*). Consiste en un conjunto de mecanismos y procesos que usan el oxígeno disuelto en el agua para promover el crecimiento biológico encargado de eliminar materia orgánica, ya sea por degradación biológica o por adsorpción [61]. Si se diseña adecuadamente, esta técnica es capaz de eliminar incluso amoniaco convirtiéndolo (oxidándolo) en nitrito primero y nitrato después (proceso denominado nitrificación), y posteriormente reduciéndolo a nitrógeno gas (desnitrificación). La biomasa generada, es separada (proceso denominado sedimentación secundaria) del agua tratada antes de ser descargada o ser conducida al tratamiento terciario. Otra técnica, no tan común, pero presente en algunas EDARs, se denomina MBR, *Membrane Biological Reactor*.

El tratamiento terciario no está incluido en muchas EDARs, pero actualmente existe una tendencia creciente en cuanto a su implementación. Aumenta la calidad del efluente al nivel requerido. En la actualidad, es usado cuando el agua va a ser vertida sobre ecosistemas frágiles (estuarios, deltas, ríos de bajo flujo, arrecifes de coral, etc.), o va a ser reutilizada para riego, recarga de acuíferos, etc. Consiste en tratamientos adicionales como filtración (por carbón activo, micro- o ultra- filtración, ósmosis inversa), eliminación de nutrientes (como nitrógeno y fósforo), desinfección (mediante cloro, UV, ozono), etc. Muchos de ellos son similares a los comúnmente utilizados en las Estación de Tratamiento de Agua Potable (ETAPs). Si se utiliza más de un proceso terciario, la desinfección es siempre la última etapa y se le denomina pulido del efluente.

Según diferentes autores [55, 62-65], la eficacia en la eliminación de fármacos durante el pre-tratamiento y el tratamiento primario es en general bastante bajo, e incluso, en algunos casos, sustancias conjugadas como metabolitos glucurónidos o sulfatos liberan los fármacos durante estas etapas [66, 67]. Es durante el tratamiento secundario donde las probabilidades de eliminación de fármacos aumentan ya sea a través de procesos de biodegradación/biotransformación y/o adsorción. La volatilización se considera poco probable en estos compuestos de naturaleza mayoritariamente muy polar. Dependiendo de

la técnica/s implementada/s en el tratamiento terciario, si lo hubiere, la eficiencia de eliminación puede mejorarse en gran medida.

Verlicchi et al. [68] han publicado recientemente una revision bibliográfica muy completa al respecto. Ésta incluye 264 EDARs de todo el planeta. En general, los influents en éstas son sometidos a un pretratamiento y a una sedimentación primaria seguida de tratamiento secunadario, que en la mayoría de ellas, en concreto en 244, consiste en CAS, y en el 20 restante, sin embargo, en MBR. La última etapa consistió en general en procesos de desnitrificación-nitrificación y eliminación de carbon, en algunos casos, en la precipitación simultánea de de fosfato mediante la adicción de sales de Fe.

Los estudios se han llevado a cabo en diferentes épocas del año, y en lugares del planeta muy dispares, cubriendo una amplia gama de posiblidades de condiciones ambientales, así como de parámetros operacionales de funcionamiento. En cuanto a estos últimos, dos de los más importantes son los llamados SRT (en ingles, Solid Retention Time) y el HRT (en ingles, Hydraulic Residence Time).

El SRT es el tiempo medio que el fango permanece en el sistema. Muchos autores, entre ellos [69, 70], han observado que un SRT largo propicia la adaptación de diferentes tipos de microorganismos, así como la presencia de especies de crecimiento lento que podrían tener una capacidad mayor para eliminar xenobióticos, además de que al mismo tiempo proporciona una mejor separación de sólidos en suspensión. Este es el caso del ibuprofeno y el diclofenaco como informó [71], cuya eliminación se logra solo tras el crecimiento de una bacteria específica. Sin embargo, esto no aparece afectar a todos los compuestos, como por ejemplo al naproxeno y al sulfametoxazol [72, 73].

El HRT es el tiempo medio que el agua está dentro del sistema. Los autores, [74] y [73], no encontraron una relación significativa entre HRT y la eliminación de diclofenaco y los beta-bloqueantes atenolol, metroprolol, acebutolol y sotalol. Sin embargo, [75] and [76] observaron que para conseguir una eliminación eficiente de un compuesto, el HRT debía ser superior a su tiempo de vida media.

A continuación se presenta una revisión pormenorizada de las eficiencias de eliminación durante el tratamiento de las EDARs, por familias terapéuticas.

En cuanto a analgésicos y anti-inflamatorios, el porcentaje de eliminación medio varía entre el 23 (tramadol) y el 99% (ácido salicílico) en CAS, y entre el 43% (indometacin) y el 99% (acetaminofén) en MBR. Para compuestos investigados en ambos sistemas, MBR siembre exhibió una mayor capacidad de eliminación que CAS. Algunos fármacos como el diclofenaco, ketoprofeno y ácido mefenámico, exhibieron un rango de variabilidad de eliminación mediante tratamientos secundarios bastante amplio.

Los antibióticos presentan un rango de variabilidad en general amplio. Las valores medios varían entre 0% (espiramicina) y 98% (cefaclor) en CAS y entre 15 (acitromicina) y el 94%

(ofloxacin) en MBRs. Solo uno de los 10 compuestos investigados, la acitromicina, ha sido investigado en ambos sistemas, proporcionando eficiencias de eliminación medias más altas en CAS [68].

Para el caso de los reguladores de lípidos y drogas psiquiátricas, la variabilidad de los rangos en las eficiencias de eliminación son también, en general, bastante amplios, pero parece observarse que existe una eficiencia de eliminación más elevada en MBRs, excepto para la carbamacepina, cuyos valores medios fueron bajos en ambos casos. Se trata no solo de uno de los compuestos más persistentes [68].

Las eficiencias de eliminación para los antihistamínicos fueron inferiores al 80%, con la excepción de la ranitidina y el valsartán. Los valores medios están entre 50 y 60%, salvo pocas excepciones como el valsartán (84% en CAS), loratadina (15% en CAS y 19% en MBR) y omeoprazol (9% en CAS) [68].

Todo lo anterior se refiere a la eliminación de fármacos de la fase acuosa, comparando simplemente el efluente con el influente, sin entrar a distinguir si la eliminación se debe a procesos de adsorción sobre el fango, y/o procesos de degradación/transformación biológica. Por esta razón, esta eliminación se califica a menudo de "eliminación aparente".

Otro enfoque consiste en considerar la EDAR como un sistema con una entrada (influente) y dos salidas (efluente y fango). Pocos autores han investigado estos dos mecanismos en detalle, proporcionando contribuciones de adsorción y biodegradación a la eliminación global (Tabla 2). De su observación se extrae que la adsorción sobre el fango no es de gran importancia para la mayoría de los fármacos, de hecho, solo los antibióticos ciprofloxacin, norfloxacin, ofloxacin y lomefloxacin, los antihipertensivos hidroclorotiacida y el regulador de lípidos fenofibrato, presentan un porcentaje de eliminación por adsorción por encima del 60%. Esto es debido fundamentalmente, a a sus características hidrofílicas. No obstante, los fenómenos de adsorción pueden venir dados también a una interacción electrostática con las (generalmente) cargas negativas de la superficie de las células de la biomasa. En este caso, el pH de trabajo de la EDAR condicionará en gran parte este último mecanismo, pero en cualquier caso, éste no varia mucho habitualmente entre EDARs [77].

1.2.2 Potabilización

Agua potable es agua lo suficientemente segura como para ser consumida sin riesgo para los humanos, es decir, es apta para el consumo humano. Posibles fuentes de agua son las aguas subterráneas, los lagos, los pantanos, los ríos, los canales, el agua atmosférica, el agua de lluvia y el agua del mar. En función de la calidad del agua utilizada, el tratamiento necesario será diferente. Normalmente, más de una técnica de purificación es usada. A escala urbana, un tratamiento tipo, ampliamente utilizado en las ETAPs, consiste en la

Tabla 2. Fracción con respecto a la carga en el efluente, de la eliminación durante el tratamiento secundario biológico, la adsorción al fango, y la no eliminación y por lo tanto descarga con el efluente, de fármacos durante el tratamiento en las EDARs.

		Transformación biológica (%)	Adsorción sobre el fango (%)	Vertido en el efluente (%)	Ref.
Analgésicos y anti-inflamatorios	**Diclofenaco**	5-45	<5	55-95	[78]
		25	<5	70-75	[79]
		10	5	85	[79]
		5	0	95	[71]
		10-30	0	70-90	[71]
	Ibuprofeno	90-100	<5	0-10	[78]
		<5	<5	95-100	[80]
		95-100	<5	0-5	[80]
		35-40	0	60-65	[71]
		95	0	5	[71]
	Indometacina	27	0	73	[79]
		40	<5	58-60	
	Ketoprofeno	70	0	30	[79]
		<95		5-10	
	Ácido mefenámico	65	7	28	[79]
		55-58	<30	<20	
	Naproxeno	55-85	<5	15-45	[78]
		77	0	23	[79]
		95-98	0	<5	[79]
		5	0	95	[71]
		85-90		10-15	[71]
Antibióticos	**Acitromicina**	<40	<10	60-90	[81]
	Cloramfenicol	0	0	100	[81, 79]
	Ciprofloxacino	<10	70-80	≤30	[82]
		<10	77	<4	[77]
	Claritromicina	<10	<5	75-90	[81]
		90	<5	10	[81]
		<10	≤10	>90	[81]
		0	18	82	[79]
		0	<45	55-60	[79]
	Enrofloxacina	19	65	17	[83]
	Eritromicina	20		80	[71]
	Lomefloxacina		60	40	[83]
	Metronidazole	15-18		100	[79, 83]
				82-85	

Antibióticos	**Norfloxacin**	<10 <10	80-90 72	≤20 <4	[82] [77]
	Ofloxacin		60	40	[83]
	Roxitromicina	<60 18	<5 2	>35 80	[81] [71]
	Sulfametacina	<85 15-18	0 20	<20 60-65	[79]
	Sulfametoxazol	50-90 20	<5 0	10-50 80	[81] [71]
	Sulfapiridina	70	<10	≥30	[81]
	Trimetoprim	<10 40 38-40 18	≤5 ≤5 <5 5-10	>90 <60 50-55 72	[81] [81] [79] [79] [71]
Antidiabéticos	**Glibenclamida**		<10 60	90-95 40	[79]
Cardiovascular drugs	**Enalapril**	95-98 95-98		2-5 2-5	[79]
	Hydrochlorotiacida		100 100		[79]
	Atenolol	<70	<5	<35	[79]
	Metoprolol	0	0 0	100	[79]
	Nadolol	35-40 70	<5 30	60	[79]
	Sotalol	10 <50	<5 <5	<90 50	[79]
	Timolol	<40 40-45	<5 0	<65 55-60	[79]
	Furosemida	35-40 75-80	<5 2-5	60-65 20	[79]
Antihistamínicos	**Cimetidina**	42 60	4 5-8	54 32-35	[79]
	Famotidina	<10 80	10 20	85 0	[79]
	Ranitidina	<20 75	<5 <5	80 20-25	[79]

Reguladores de lípidos	**Bezafibrato**	12 <80 45-50	2 <5 <5	86 20-25 50	[79] [79] [80]
	Fenofibrato	0 25-30	100 65-70	0	[79]
	Gemfibrocilo	0 90	3 <5	97 5-10	[79] [79]
	Pravastatina	45 62	0 2	55 <40	[79]
Drogas psiquiátricas	**Carbamacepina**	<40 22 0	<5 3 5	>60 75 95	[78] [79] [79]
	Diacepam	0	42 65	58 35	[79]
	Fluoxetina	80 90	0 0	20 10	[71] [71]
	Loracepam	30 30	<5 5-8	65-70 65	[79]
Beta-agonistas	**Salbutamol**	<60 40-42	<5 2	<45 55-60	[79]

sucesión de las siguientes etapas: sedimentación, coagulación/floculación, filtración y desinfección [84].

Durante la sedimentación se produce el asentamiento por gravedad de las partículas sólidas contenidas en el agua. Se realiza en depósitos anchos y de poca profundidad y, cuanto mayor sea el tiempo de reposo, mayor será el asentamiento, y consecuentemente menor la turbiedad. El reposo prolongado también ayuda a mejorar la calidad del agua debido a la acción del aire y los rayos del sol, que oxidan el hierro y eliminan algunas substancias.

En la mayoría de los casos, el agua contiene material en suspensión, que puede ser de origen orgánico o inorgánico, y que no precipita ni tras un reposo prolongado. En muchos casos, se trata de coloides y son la causa principal de la turbiedad. Cada partícula en un coloide se encuentra estabilizada por cargas de igual signo sobre su superficie, repeliendo a las partículas vecinas, lo que impide su aglomeración. Las operaciones de coagulación y floculación desestabilizan estos coloides y consiguen su sedimentación. Esto se logra, por lo general, con la adición de agentes químicos. Algunos de los coagulantes más comúnmente utilizados son sulfato de aluminio o cloruro férrico. La coagulación, por lo tanto, es la desestabilización del coloide mediante la eliminación de las capas eléctricas que rodean a

todas las partículas coloidales. Y la floculación es la aglomeración de partículas desestabilizadas susceptibles de precipitar por gravedad.

El agua sobrenadante es ahora filtrada. Existen varios tipos de filtros, los más comunes, son los llamados filtros rápidos, en los que el agua pasa verticalmente a través de una capa de arena que a menudo tiene una capa de carbón activo en la superficie. Hoy en día existen también técnicas denominadas ultrafiltración y nanofiltración que usan membranas poliméricas con poros micro- y nanoscópicos, respectivamente, capaces de separar del agua sustancias disueltas. La ósmosis reversa, puede incluso separar iones inorgánicos. Así, es una de las técnicas utilizadas en las plantas desaladoras.

Por último, la desinfección, tiene como función eliminar los patógenos (virus, bacterias y protozoos) que no han sido eliminados en las etapas anteriores, así como proporcionar una dosis residual de desinfectante durante el almacenaje y sistema de distribución. Existen muchos sistemas de desinfección, pero algunos de los agentes más comunes son la radiación ultravioleta (UV), el ozono y el cloro. Todos tienen ventajas e inconvenientes. Con los dos primeros el riesgo de obtención de subproductos cancerígenos es menor o ausente en comparación con la cloración. No obstante, éste último es el más barato, y además mantiene una actividad latente que asegura un agua desinfectada durante el almacenaje y distribución hasta que llega a los grifos.

Se sabe mucho menos del comportamiento de los fármacos en las ETAPs que en las EDARs [10]. Esto es debido fundamentalmente a que se encuentran a concentraciones cercanas a los límites de detección y cuantificación de los métodos de análisis y, por lo tanto, resulta difícil sacar conclusiones. Así, la mayoría de los estudios son a escala de laboratorio. El porcentaje de eliminación de los fármacos depende tanto de la naturaleza de éstos como de la técnica/s de tratamiento utilizada/s. Además, debe también tenerse en cuenta, la posibilidad de formación de productos de transformación tóxicos durante el tratamiento. A continuación se indican la eficiencia de eliminación de fármacos asociada a algunas de las técnicas comúnmente utilizadas en las ETAPs:

Filtración por carbón activo:
Es eficiente para la eliminación de microcontaminantes entre lo que se incluyen los fármacos, pero también pesticidas y estrógenos [85-88]). Las interacciones hidrofóbicas son el mecanismo dominante en la eliminación de la mayoría de compuestos orgánicos en los sistemas de adsorción por carbón activo. Como resultado, el carbón activo elimina eficientemente la mayoría de los compuestos orgánicos no polares, es decir, con log K_{ow} >2. No obstante, la materia orgánica presente en el agua compite por los sitios de adsorción y disminuye la capacidad del carbón activo para eliminar los microcontamintes [89-93].

La filtración con carbón activado granular (GAC) fue un procedimiento muy efectivo en [94] en la eliminación de productos farmacéuticos, excepto para el ácido clofíbrico.

Ozonación:
Para el caso del ozono, debido a su naturaleza selectiva, la transformación de microcontaminantes puede requerir el uso de procesos avanzados de oxidación (*Advanced Oxidation Processes*, AOPs), tales como UV/peróxido de hidrógeno ([95, 96]). Algunos estudios han documentado la eliminación de fármacos por ozonación en tratamientos a escala piloto, como por ejemplo diclofenaco, carbamacepina y bezafibrato, pero no ácido clofíbrico ([88]). La eliminación mejoraba cuando la ozonación se llevaba a cabo en presencia de peróxido de hidrógeno (0.4-0.7 mg H_2O_2/mg ozono dosificado) [10].

[94] encontraron la ozonazión bastante efectiva en la eliminación de fármacos polares. Las concentraciones de diclofenaco y carbamacepina se reducían en más del 90%, el bezafibrato en un 50%, mientras que el ácido clofíbrico se mantuvo estable incluso a altas dosis de ozono.

Radiación UV:
En ocasiones ha sido utilizada en solitario para el tratamiento de microcontaminantes [10], pero las dosis necesarias son varios órdenes de magnitud más elevadas que para la desinfección microbiana. Esto la pone en desventaja económicamente con otros métodos de tratamiento como la ósmosis reversa. No obstante, por citar algunos datos de su eficacia, dosis de 3000 mJ/cm^2 eliminaron entre el 50-80% de siete antibióticos en agua destilada y natural.

Biotransformación
Se conoce poco acerca de la importancia de la biotranformaicón en los tratamientos de potabilización. No obstante, durante filtración lenta por arena en DWTPs estudiadas por [88], muchos de los fármacos fueron eliminados, pero otros compuestos recalcitrantes como los antiepilépticos carbamacepina y primidona persistieron.

En [94] la filtración por arena bajo condiciones aeróbicas y anóxicas, no eliminó significativamente los fármacos estudiados.

Separación por membranas:
La mayoría de los fármacos presentan un tamaño molecular entre los 150 y 500 Da. Como consecuencia, solo los compuestos asociados a partículas o coloides se eliminarán mediante microfiltración y ultrafiltración. Así por ejemplo, la microfiltración no eliminó los betabloqueantes en los estudios llevados a cabo por [97]. Sin embargo, la mayoría de los fármacos sí se eliminarán por ósmosis reversa y nanofiltración. Así por ejemplo, la ósmosis reversa puede lograr eliminaciones por encima del 90% de hormonas esteroides [97]. Por otro lado, compuestos polares y cargados se eliminarán mejor que los neutros o poco polares, debido a la naturaleza cargada de las superficies de las membranas.

1.3 Legislación medioambiental de fármacos

Debido a que han sido reconocidos como contaminantes recientemente, hay mucho por desarrollar en materia legislativa en cuanto a presencia, control y seguimiento de fármacos en el medioambiente, así como en las aguas residuales, sus principales fuentes de entrada [8].

En la actualidad, y centrándonos en la Unión Europea, la *Directiva Marco del Agua 2000/60/CE* marca como objetivo para el 2015, alcanzar un mejor estado ecológico y químico de las aguas continentales europeas (aguas superficiales y subterráneas), mediante la adopción de medidas encaminadas a reducir, limitar y prevenir la contaminación. Seis años más tarde, apareció una directiva semejante a la marco, pero desarrollada específicamente para la protección contra la contaminación y deterioro de las aguas subterráneas europeas. Esta es la *Directiva 2006/118/EC*. En 2008, una enmienda a la directiva marco, la *Directiva 2008/105/EC*, publicó valores límite permitidos en las aguas continentales superficiales a una lista de 33 sustancias prioritarias, entre las que se encontraban metales pesados, pesticidas y retardantes de llama. En enero de 2012, se publicó una propuesta de nueva enmienda a la directiva marco, sugiriendo la ampliación de compuestos prioritarios a controlar en las aguas continentales europeas con otros 15 compuestos, entre los que se encuentran dos hormonas sexuales, el 17-α-etinil-estradiol, y el 17-β-estradiol, y por primera vez un fármaco, el analgésico/anti-inflamatorio diclofenaco. En cuanto a agua marina, en 2008 se publicó la *Directiva 2008/56/EC*, similar a la directiva marco, pero en este caso en el campo de la política medioambiental marina. Destacar aquí, que la lista de sustancias prioritarios publicada para las aguas continentales superficiales en la directiva marco, es aplicable también a ésta. Actualmente, no existe legislación medioambiental sobre fármacos en otros compartimentos medioambientales, como terrestre (sedimentos o suelos) o biota, que considere a los fármacos como una amenaza.

La entrada de fármacos en el medioambiente, estaría controlada en gran medida, si existiese legislación en materia de aguas residuales que incluyese a los productos farmacéuticos. Pero hasta el momento, la legislación vigente acerca del tratamiento de aguas residuales urbanas, la *Directiva 91/271/ECC*, solo exige controles en cuanto a DBO, Demanda Química de Oxígeno (DQO), sólidos en suspensión, y presencia de compuestos inorgánicos de nitrógeno y fósforo. A nivel estatal, existe un Real Decreto, el *R.D. 1620/2007*, que regula la reutilización de las aguas residuales tratadas, como riego, por ejemplo. Pero ni siquiera en este caso, se controlan los fármacos. Tampoco son contemplados por la *Directiva 86/278/EEC*, destinada a la protección de los suelos agrícolas abonados con fangos de depuradora. Las aguas residuales industriales, y entre ellas las de las industrias farmacéuticas, así como las generadas por los hospitales, son habitualmente vertidas a la red de saneamiento urbana, conduciéndolas hacia las EDARs municipales, donde son tratadas como aguas residuales urbanas. Cuando esto no es posible y la industria tiene que verter directamente en un agua medioambiental, necesita que el correspondiente

organismo competente en el país miembro, le otorgue un permiso de vertido, tras cumplir una serie de requisitos, establecidos en España en el R.D. de Dominio Público Hidráulico, entre los que el contenido en fármacos no se encuentra. Tampoco se encuentra nada en cuanto a lixiviados de basureros.

Un control realmente eficaz sobre el origen de estos contaminantes se produciría con la existencia de legislación verdaderamente comprometida con el medioambiente, sobre la producción de medicamentos, así como en el desarrollo de nuevos productos, y su posterior venta y consumo. Actualmente, existen la *Directiva 2001/83/EC* (con sus posteriores modificaciones, la *Directiva 2004/27/EC* y la *Directiva 2010/84/EU*), y la *Directiva 2001/82/EC*, relativas a fármacos de consumo humano y veterinario, respectivamente, que establecen que las peticiones de autorización de comercialización de nuevos fármacos, deben incluir una valoración de potenciales riesgos medioambientales generados por el producto farmacéutico. Se trata de una medida muy pobre, puesto que además de no incluir a los fármacos ya autorizados, en el caso de los fármacos de consumo humano, cualquiera que sea el impacto medioambiental previsto, esto no supone un impedimento en la obtención de la autorización. Para el caso de los fármacos veterinarios, si el informe sugiere amenaza al medioambiente, los estados miembros tendrán potestad para suspender su comercialización y uso en su territorio.

El reglamento europeo de 2006 REACH (*Registration, Evaluation, Authorisation and Restriction of Chemicals*), está dirigido a la producción y el uso de cualquier sustancia química, y sus impactos potenciales tanto en los seres humanos, como en el medio ambiente en general. Se trata de una normativa exigente, que ha surgido en un intento de frenar el uso, un tanto incontrolado durante los últimos años, de sustancias químicas. No incluye, no obstante, a los fármacos, por estar éstos ya sometidos a legislación propia.

Como se ha comentado en secciones anteriores, los fármacos que están llegando a las aguas potables, sin embargo, no sufren ningún control legislativo. La *Directiva 98/83/EC*, sobre la calidad del agua pretendida para el consumo humano, es la que sirve de referencia en España, y no incluye el control de estos contaminantes emergentes. Tampoco en el Real Decreto 1798/2010, que regula la explotación y comercialización de aguas minerales naturales y aguas de manantial envasadas para consumo humano.

1.4 Proceso analítico de determinación de contaminantes farmacéuticos en agua

Es común el análisis de la contaminación del agua en magnitudes convencionales como la turbidez, DQO y DBO, concentración de metales pesados, radiactividad, dureza, contenido en nitratos, sulfatos, etc. En muchos casos, estas magnitudes están legisladas en las aguas ambientales, residuales y/o de bebida. El gran avance de la química analítica ha sido el progreso desarrollado con las nuevas técnicas, las cuales permiten la detección de diversos

contaminantes orgánicos, a nivel traza. No hace mucho tiempo que con la instrumentación disponible, no se podían conseguir límites de sensibilidad necesarios para detectar concentraciones individuales de la mayoría de los contaminantes, o al menos de los denominados microcontaminantes. En cambio, actualmente, se puede llegar a límites del orden de ng L^{-1}, e incluso más bajos. Para el caso del análisis de fármacos como contaminantes emergentes, este hecho está estrechamente vinculado con los avances en la instrumentación en el campo de la espectrometría de masas (*Mass Spectrometry*, MS*),* especialmente de la espectrometría de masas en tándem (MS/MS).

Las primeras determinaciones analíticas de fármacos no fueron en el campo de la química ambiental, sino en el desarrollo farmacéutico, para el análisis de pureza [98], o control de farmacocinéticas [99-103]. También fueron pioneros los controles de fármacos en alimentos [104]. Stumpf et al. [105] fueron los primeros en identificar diclofenaco, ibuprofeno, ácido acetilsalicílico y ketoprofeno en agua residual y río. Desde entonces la publicación en literatura científica de metodología analítica para la determinación de fármacos en matrices ambientales ha ido en aumento [106]. Los primeros métodos desarrollados, se centraban en clases terapéuticas específicas [107-113], con los antibióticos siendo las más estudiadas debido a la preocupación sobre su potencial para provocar resistencias [114-119]. Sin embargo, si se requieren los resultados de muchos parámetros, el uso de métodos individuales consume mucho tiempo. Actualmente, la tendencia se centra en el desarrollo de métodos genéricos, los cuales permiten el análisis simultáneo de una amplia variedad de fármacos pertenecientes a diversos grupos terapéuticos. Las fármacos más frecuentemente incluidos en tales métodos multi-residuos, pertenecen a los analgésicos y anti-inflamatorios, antibióticos, reguladores de lípidos, drogas psiquiátricas, beta-bloqueantes y hormonas sexuales [106]. En la actualidad, la comunidad científica reclama más investigación sobre los procesos de degradación y el destino de los productos de transformación en el medio ambiente. Además, ciertos procesos de desinfección aplicados en las plantas de tratamiento (cloración, ozonización, UV, etc.) potencialmente cambian la valoración del riesgo en el consumo humano de los compuestos padre a los productos de degradación. En consecuencia, se requiere también el desarrollo de protocolos analíticos genéricos que permitirán simultáneamente la determinación de compuestos padre y sus productos de transformación. También es común, que en un mismo método multi-residuo, se incluyan también otros contaminantes no farmacéuticos, como productos de cuidado personal. De esta manera, estos métodos son herramientas que proporcionan un conocimiento más amplio sobre la presencia de contaminantes en el medio ambiente, lo que es útil y necesario para realizar estudios posteriores de eliminación, distribución y destino final en el medio ambiente [106]. Además, se prefieren cada vez más los métodos automatizados, los cuales permiten conseguir límites de detección más bajos, con la menor manipulación de muestra posible.

Debido a la polaridad de la gran mayoría de fármacos y de su escasa volatilidad, cabe esperar que sean las aguas ambientales la matriz ambiental que albergue la mayor cantidad

de contaminantes farmacéuticos. Así, la mayoría de las metodologías ambientales para fármacos publicadas son aplicables para aguas subterráneas y superficiales. En muchos casos, éstas son también aplicables sobre aguas residuales y potables.

1.5 Justificación y objetivos de la tesis

Los fármacos, compuestos químicos con actividad biológica, y sus productos de transformación, metabolitos y otros derivados en las plantas de tratamiento y en el medio ambiente, con actividad farmacológica remanente, son contaminantes antropogénicos que, en muchos casos, están llegando al medioambiente a través de las aguas residuales, y son las aguas superficiales el primer y principal compartimento ambiental destino.

Este es un hecho que, en la actualidad, es ya ampliamente reconocido por la comunidad científica, tras el desarrollo de métodos de análisis capaces de la determinación de estos microcontaminantes en el medio ambiente, y su posterior aplicación en estudios de presencia en diferentes matrices ambientales. Los científicos, por lo tanto, están demandando medidas de control de los mismos por parte de las autoridades. Aunque tímidos, se están dando pasos adelante en materia legislativa en la lucha contra este tipo de contaminación, lo que muestra la toma de conciencia de la importancia en el control de las fuentes de entrada de estos contaminantes emergentes, así como de su eliminación del medioambiente.

Esto conlleva un aumento importante en el número de estudios y, por lo tanto, en las muestras a procesar por parte de los laboratorios medioambientales. De ahí nace la necesidad de optimizar la eficiencia y competitividad de los métodos analíticos actuales, de manera que progresen en *fiabilidad* y *sensibilidad*, aumentando al mismo tiempo su *versatilidad*, así como su *capacidad* y *rendimiento*. El desarrollo tecnológico en el sector es muy rápido, y en general, es la comunidad científica la que evalúa en primer término la viabilidad y bonanza de las diferentes propuestas tecnológicas.

Con la presente tesis se ha pretendido dar respuesta a estas necesidades. Para ellos, se plantean los siguientes objetivos:

- Desarrollar y validar métodos analíticos para la determinación de fármacos y sus derivados en aguas medioambientales y residuales, mostrando las ventajas y puntos débiles de cada uno de ellos. En concreto, se pretende utilizar dos fuentes de *fiabilidad*, que no son excluyentes y pueden ser utilizadas simultáneamente. La primera consiste en la introducción de la automatización en el tratamiento de muestra, llevando al mínimo la manipulación de las muestras por parte del analista. La segunda consiste en la introducción de surrogados y patrones internos adecuados que pongan de manifiesto las incidencias producidas durante el proceso analítico, para que puedan ser corregidas. Por otro lado, se

propone conseguir métodos que presenten una elevada *sensibilidad* para la determinación de los microcontaminantes objeto de estudio presentes a bajas concentraciones en matrices complejas. Para ello se pretende utilizar espectrómetros de masas de triple cuadrupolo de última generación, que trabajando en modo SRM (*Selected Reaction Monitoring*), proporcionan una muy buena sensibilidad, además de una elevada selectividad, que allanan el camino de la *fiabilidad* comentada anteriormente. Además, se quiere apostar también, por el desarrollo de métodos con la suficiente *versatilidad* como para que puedan ser aplicados sobre cualquier tipo de agua ambiental dulce continental, tanto superficial como subterránea, así como a agua residual y potable, con el fin de propiciar la intercalación de muestras de diferente naturaleza analizadas consecutivamente en una misma tanda. Finalmente, se quiere proponer métodos dotados de una elevada *capacidad*, de manera que un solo método sea capaz de analizar el mayor número de residuos de interés posibles, incluyendo fármacos y sus derivados, metabolitos y otros productos de transformación. Pero a la vez, se pretende conseguir un aumento en el *rendimiento* analítico de estos métodos, es decir, lograr una disminución del tiempo de análisis por muestra y un aumento del número de muestras por día. Para esto se quiere apostar claramente por la automatización, eliminando algunas etapas en el proceso analítico, lo que previsiblemente, además de redundar en fiabilidad como se ha comentado anteriormente, puede proporcionar métodos analíticos más cortos y autónomos y, por lo tanto, capaces de trabajar en ausencia del analista. Otra propuesta en esta dirección fue la utilización de cromatografía rápida. En el capítulo 2 de la presente tesis se discuten los logros conseguidos al respecto.

- Otro objetivo importante de la presente tesis, es la aplicación posterior de los métodos desarrollados, en estudios medioambientales de elevado interés científico por su innovación. En concreto en los siguientes:

- Estudio del impacto medioambiental a escala real, en un escenario de reutilización de agua residual para el mantenimiento del caudal ecológico de un río mediterráneo. Además, se pretende valor el riesgo ecotoxicológico y de estrogenicidad en las muestras del río.
- Estudio de presencia de fármacos y sus productos de transformación en las aguas superficiales de la cuenca del río Ebro.
- Estudio de la distribución tridimensional y origen de residuos farmacéuticos (fármacos y sus productos de transformación) en las aguas subterráneas bajo la ciudad de Barcelona (España).
- Determinación enantiomérica de 13 residuos farmacéuticos quirales en las aguas superficiales de la cuenca del río Guadalquivir, así como en las aguas de entrada y salida a las principales EDARs asociadas a la cuenca. Además, se quire discutir las implicaciones en la toxicidad de las relaciones racémicas y no racémicas.

Los hallazgos encontrados en estos cuatro estudios de presencia de fármacos están discutidos en el capítulo 3 de la presente tesis.

Esta tesis se ha estructurado en tres apartados:

- Una introducción que incluyen los antecedentes a la tesis, donde se describen la figura de los fármacos y sus derivados como contaminantes del medio ambiente acuático, las actuaciones para la minimización de su entrada en las aguas medioambientales y su existencia en el agua de bebida, la legislación actual relacionada con la introducción y presencia de estos compuestos en el medio ambiente, así como el proceso analítico de determinación de estos compuestos en matrices acuosas.
- El capítulo 2 está dedicado al desarrollo de métodos de análisis de residuos farmacéuticos en agua, haciendo una descripción de cada una de las etapas del proceso. Se presenta, además, la descripción del desarrollo de cuatro nuevos métodos innovadores.
- En el capítulo 3 se han aplicado los métodos desarrollados en el capítulo 2 en cuatro estudios determinísticos de fármacos y sus productos de transformación, en aguas medioambientales y residuales, que arrojan luz sobre la situación de contaminación hidrológica por estos compuestos en la Península Ibérica.

Finalmente se incluyen las conclusiones generales obtenidas en el trabajo realizado en esta tesis, así como la bibliografía correspondiente.

2. DESARROLLO DE MÉTODOS DE ANÁLISIS DE FÁRMACOS EN AGUA

2.1 Introducción

El proceso analítico de determinación de contaminantes farmacéuticos en agua contiene, de manera general, las siguientes etapas: 1) Muestreo, 2) Pre-tratamiento de muestra (preconcentración y aislamiento) y, 3) Análisis instrumental.

Tras el desarrollo y optimización de este proceso, y antes de ser utilizado como método analítico de muestras reales, deben comprobarse sus parámetros de validación.

2.1.1 Muestreo

La principal finalidad del muestreo es conseguir muestras representativas de la matriz a determinar, así como mantener su integridad hasta el momento del análisis.

Existen dos maneras de proceder durante la toma de muestra: discreta o compuesta. La primera consiste en una toma individual, una muestra aislada del punto de muestreo. Ésta proporciona una visión instantánea de la concentración de contaminantes en el momento de toma de muestra. En la segunda, en cambio, la toma de muestra consiste en la acumulación de volúmenes de muestra de un mismo punto de muestreo a lo largo del tiempo, mezclándolos en un mismo recipiente.

En las aguas residuales, es común que la concentración de los contaminantes fluctúe mucho con el tiempo, con variaciones estacionales (invierno y verano), semanales (entresemana y fin de semana), e incluso diario (día y noche). Las aguas superficiales también lo hacen, pero su variación está más ralentizada y/o no es tan frecuente. Es por eso que, generalmente, en aguas medioambientales, se tomen muestras discretas, mientras que en aguas residuales de EDARs o potables de ETAPs se recojan muestras compuestas, en concreto lo más común es recoger, mediante muestreadores automatizados, volúmenes de agua idénticos a intervalos de tiempo constantes durante 24h.

En cuanto a la estabilidad y conservación de la muestra hasta el momento del análisis, lo que se procura es reducir o eliminar al máximo cualquier proceso que altere la presencia de los contaminantes en las muestras (degradación, transformación, adsorción, etc.). La refrigeración puede ser insuficiente [120], siendo la congelación (normalmente a -20ºC) tras la filtración por 0.45 µm, una de las prácticas más comunes para ralentizar la actividad microbiana, que puede degradar los contaminantes objeto. Otras prácticas también utilizadas son la adicción de un 1% de formaldehido, acida sódica, o bajar el pH a 2-3 con ácido clorhídrico o sulfúrico para inhibir los procesos de degradación microbiana. Para evitar degradación por radiación UV es necesario protegerlas de la luz. Para ello, es recomendable almacenarlas en recipientes opacos o de color ámbar, y en cualquier caso, mantenerlas en oscuridad siempre que sea posible. Además, el material de los recipientes y demás instrumental en contacto con la muestra no debe interferir en la composición de contaminantes, ya sea por adsorción o por reacción con sus componentes. Así, los filtros

más comúnmente utilizados son de fibra de vidrio, nailon o politetrafluoroetileno (PTFE), también conocido como teflón. En cuanto a los recipientes, lo más habitual es el uso de botellas de tereftalato de polietileno (*Polyethylene terephthalate*, PET), PTFE o vidrio. En el último caso, se recomienda su silanización previa, puesto que algunos compuestos como los beta-bloqueantes, los protectores de estómago y algunos antibióticos, como las tetraciclinas y macrólidos, tienen tendencia a adsorberse sobre sus paredes [121]. Una buena práctica además, consiste en el llenado completo de las botellas evitando dejar burbujas de aire, minimizando la probabilidad de reacciones de oxidación.

En cualquier caso, la práctica más segura es el proceder con el análisis de las muestras a la mayor brevedad posible tras su toma.

2.1.2 Pre-tratamiento de muestra (preconcentración y aislamiento)

Como se ha comentado antes, la filtración por 0.45 µm, es una técnica habitual realizada a la mayor brevedad posible tras la toma de muestra, para eliminar microbios y ralentizar así la biodegradación. Con esta técnica, además, se elimina todo el material en suspensión, no disuelto en el agua. Los fármacos son, en general, hidrofílicos, por lo que se espera que se encuentren en su mayoría en la fase disuelta y, por lo tanto, poco retenidos en las partículas. No obstante, una opción para arrastrar la fracción de analitos retenidos en el particulado, es añadir una pequeña cantidad de metanol (MeOH) sobre el filtro utilizado, y mezclar el extracto con el resto del agua filtrada. Otra opción, es analizar separadamente los fármacos adsorbidos sobre las partículas retenidas en el filtro, y saber así la fracción de compuestos presentes en los sólidos en suspensión frente a los disueltos en el agua.

A continuación, y centrándonos en la fase disuelta, debido a los niveles de ultra-traza que se esperan (entre ng L^{-1} y µg L^{-1}) para estos microcontaminantes [108], un procedimiento de preconcentración es casi siempre necesario para alcanzar los niveles de sensibilidad deseados, incluso haciendo uso de los detectores más sensibles a día de hoy [122]. Además, para cualquier matriz medioambiental, pero especialmente si éstas son complejas, como las aguas residuales, una etapa de limpieza o eliminación de componentes interferentes (*cleanup*), es también necesaria. Existe una técnica, la extracción en fase sólida (*Solid Phase Extraction*, SPE) que posibilita la realización simultánea de ambas tareas y se ha convertido en la técnica por excelencia para aislar y enriquecer fármacos en muestras medioambientales acuosas [106, 123], reemplazando casi completamente la extracción líquido-líquido debido a sus conocidos inconvenientes [124]. A esto ha contribuido también la gran oferta de sorbentes disponible en el mercado, que cubren un amplio rango de propiedades de los analitos, incluyendo los analitos polares [125-128]. Se pueden encontrar materiales selectivos o genéricos. La elección de unos u otros depende, respectivamente, de si se pretende extraer una sola sustancia o grupo de sustancias con estructura análoga, o si

por el contrario, el fin es la extracción cuantitativa de compuestos con propiedades físico-químicas dispares. Buscando simplificación, la tendencia actual se centra en el uso de un único cartucho tipo genérico capaz de extraer en una sola etapa todos los analitos de interés del método multi-residuo, dejando de lado antiguos hábitos de combinación de diversos cartuchos. Aunque el rendimiento de los cartuchos genéricos ha mejorado mucho, es habitual el tener que establecer un compromiso en la elección de los parámetros óptimos de extracción, que pueden no ser los mejores para todos los analitos estudiados [106]. Así por ejemplo, Castiglioni y colaboradores [129] consiguieron porcentajes de recuperación tras una SPE simultánea de 30 fármacos pertenecientes a diversos grupos terapéuticos en aguas residuales urbanas, que variaron entre 36 y 131%, y Gómez y colaboradores [130], para 16 fármacos en efluentes de hospitales, presentaron porcentajes variando entre 45 y 112%. Otro inconveniente de los sorbentes genéricos es que también extraen eficientemente componentes de la matriz, empeorando, por lo tanto, su función en el *cleanup*, lo que puede tener consecuencias sobre la detección en espectrometría de masas, si ésta se realiza con la fuente de ionización de electrospray. Así, cuanto más selectivo es el sorbente, más eficiente será el *cleanup*. Un nivel de selectividad muy alto es conseguido con los denominados sorbentes MIPs (*Molecular Imprinted Polimers*), materiales diseñados a medida que pretenden la extracción cuantitativa y en exclusiva, de los analitos de interés [131].

Existen varias publicaciones que muestran estudios de evaluación de diferentes fases estacionarias de SPE en la extracción de diferentes fármacos sobre matrices acuosas, [132, 133]. Sin embargo, en ocasiones, como se apunta en [134], éstas llevan a conclusiones contrarias con respecto al mejor material sorbente a utilizar para la extracción de un mismo grupo de compuestos farmacéuticos. Así por ejemplo, para anti-inflamatorios no esteroidales, algunos autores indican que los sorbentes de sílica C18 presentan un porcentaje de recuperación superior que los sorbentes poliméricos [132], mientras otros reportan rendimientos mayores para los cartuchos poliméricos Oasis HLB [110, 113]. Para fármacos de polaridad media y alta, muchos autores usaron los sorbentes Oasis MCX, que incluyen mecanismos de intercambio catiónico y de fase invertida.

Una variante de la SPE es la microextracción en fase sólida (*Solid Phase Micro Extraction*, SPME), que puede ser utilizada también como técnica de muestreo, y que está siendo cada vez más utilizada para aislar y preconcentrar fármacos en muestras medioambientales [135-139].

En cualquier caso, se sabe que las etapas de análisis que consumen más tiempo son el muestreo y la preparación de muestra, estimándose un total de más del 80% del tiempo de análisis [140] invertido en ellas. De hecho, se ha reconocido la preparación de muestra como el principal cuello de botella en el proceso analítico en el análisis de componentes traza [52]. Un tiempo de análisis por muestra elevado, limita el número de muestras que un laboratorio puede afrontar. Por lo tanto, en los últimos años, se tiende a la automatización

de esta fase. Esto redunda en muchos casos, en un mayor rendimiento, ya que, en la mayoría de los casos, los equipos automáticos pueden trabajar largos periodos desatendidos, dejándoles trabajando por ejemplo también por la noche, con lo que el aprovechamiento del tiempo es mucho mayor. Constituyen también, por lo tanto, una mayor comodidad y seguridad sanitaria para el analista, además de que proporcionan una mayor robusted al método, con menos probabilidad para la introducción de errores o contaminación por manipulación, obteniéndose métodos con mayor reproducibilidad. Un ejemplo de instrumento que lleva a cabo SPE automatizada es el Gilson Aspec, aplicado por ejemplo por Kasprzyk-Hordern y colaboradores [141]. Un paso más ha sido ya, la conexión en línea de técnicas de pretratamiento automatizadas, a la posterior separación y detección instrumental, lo que constituye los denominados métodos online, descritos a continuación.

2.1.2.1 Métodos online

La SPE es la técnica para la que más desarrollo instrumental online se ha producido. En la mayoría de los casos, este acoplamiento se realiza sobre instrumentos de cromatografía de líquidos (LC, *Liquid Chromatography*) de alta eficacia (*High Performance Liquid Chromatography*, HPLC) con detección por espectrometría de masas en tándem (MS/MS), siendo los triples cuadrupolos (QqQ) los analizadores de masas más comunes. Es decir, SPE-LC-MS/MS. Existen, no obstante, también en el análisis medioambiental, sistemas acoplados a cromatografia de gases (Gas Chromatography, GC). Con tales acoplamientos, se alcanza un grado de automatización muy elevado, reduciéndose la intervención del analista a las etapas de filtrado, y si es el caso, de ajuste de pH o adicción de algún complejante para mejorar el rendimiento de la SPE. Con los métodos online, el total de la muestra extraída sobre el cartucho de SPE, es posteriormente eluida directamente hacia la columna cromatográfica. Por lo tanto, tamaños de muestra más pequeños son suficientes para obtener el mismo factor de preconcentración que en los sistemas desacoplados (los denominados métodos offline), donde solo una pequeña proporción del eluato es inyectado en los sistemas cromatográficos [142]. Así, de los cientos o incluso miles de mL requeridos en los métodos offline [143-145], solo unas pocas unidades de mL (normalmente entre 1 y 5 mL) son necesarios con los métodos online. Esto proporciona grandes ventajas de almacenamiento, muy bienvenidas en los laboratorios de análisis de rutina. Además, el acoplamiento online de la SPE supone la simplificación del proceso de SPE, con la eliminación de muchas de las etapas presentes en el procedimiento offline, como la evaporación del eluato para la posterior reconstitución.

Los métodos online son también considerados más respetuosos con el medioambiente. Debido a la reducción en el tamaño de muestra y a la eliminación de etapas del proceso en comparación con los métodos offline, los métodos online consumen menores cantidades de disolvente [146] tanto en la extracción como en el posterior lavado del material utilizado, así

como de otros productos químicos (agentes complejantes, ácidos y bases para ajustar el pH, etc.), lo que también repercute positivamente en la salud del personal del laboratorio. No hay que olvidar tampoco, la reducción en los recipientes y otros materiales como filtros, etc., utilizados durante el muestreo y el análisis. Todo esto supone ya en sí mismo, una reducción de costes, pero también una reducción en la cantidad de residuos generados, con el consiguiente ahorro en su gestión [52].

Además, algunos sistemas permiten la elución, separación cromatográfica y detección de una muestra al mismo tiempo que llevan a cabo la extracción de la muestra siguiente [147]. Esto conlleva un ahorro de tiempo sustancial, un aumento en el rendimiento del proceso analítico a sumar al ya conseguido con la automatización desacoplada. Esta disminución del tiempo de análisis por muestra reduce también el riesgo de evaporación de la muestra o de degradación de los analitos durante el proceso analítico [52].

Por lo tanto, esta optimización basada en la automatización, simplificación y cambio de escala presenta, muchas ventajas en términos económicos a largo plazo, técnicos y de seguridad laboral. Como inconvenientes se pueden, no obstante, nombrar que se trata de una técnica destructiva, y puesto que todo el eluato es conducido a la columna cromatográfica, solo permite analizar el extracto de la muestra una vez [147]. En los sistemas offline, sin embargo, puesto que solo una pequeña parte (en general, 10 o 20 µL) del total del extracto (en general, 0.5 o 1 mL), es inyectada en el sistema cromatográfico, el resto queda disponible para futuras réplicas si fuese necesario. Además la mayoría de los sistemas online presentan menos flexibilidad, no permitiendo combinar cartuchos de extracción. En cualquier caso, quizás el mayor inconveniente sea que se trata de un equipamiento caro.

En la práctica, el acoplamiento online más común de SPE con LC, consiste en la implementación de una pequeña precolumna dentro del loop de una válvula rotatoria de seis puertos. Mientras se lleva a cabo la separación cromatográfica y el análisis de una muestra, la siguiente muestra está siendo cargada en la precolumna por medio de una segunda bomba de alta presión. Cuándo el análisis de la primera muestra termina, la válvula gira y la fase móvil cromatográfica pasa a través del sorbente donde se extrajo la segunda muestra, y eluye y transfiere los analitos hacia la columna analítica. Una segunda alternativa consiste en colocar la columna SPE fuera de la válvula de seis puertos. Con esta configuración, los analitos retenidos se desorben con un volumen discreto de disolvente, y son posteriormente transferidos al loop de inyección. La principal desventaja de ambas opciones de online SPE-LC es el progresivo deterioro de material de extracción al ser reutilizable y el riesgo de contaminación cruzada cuando se analizan muestras altamente contaminadas [124]. Por lo tanto, en un intento de encontrar métodos más robustos y fiables, se han desarrollado sistemas de cartuchos intercambiables. Así, en los últimos años, han salido a la venta varias generaciones de procesadores de muestras con columnas sorbentes de usar y tirar, como Prospekt, Prospekt-2 y Symbiosis de la casa comercial Spark

Holland, y LiChrography OSP-2 de Merck. No obstante, aunque usan cartuchos desechables, pueden ser reutilizados como demostraron [148] y [149] con 20 y 8 inyecciones consecutivas, respectivamente, sin observar variación en las recuperaciones. La publicación científica 2 incluída en el presente capítulo, presenta el desarrollo de un método para la determinación de fármacos mediante SPE acoplada a LC-MS/MS haciendo uso de un Spark Holland Simbiosis Pico.

Algunos ejemplos de métodos online no basados en la SPE, sino en otras técnicas de extracción son la LLME (*holow fibre Liquid-Liquid Membrane Extraction*), SLME (*Supported Liquid Membrane Extraction*) [150] y la SPME [151]. La publicación científica 3 incluída en el present capítulo, presenta el desarrollo de un método para la determinación de fármacos en aguas ambientales mediante una técnica online de preparación de muestra denominada TurboFlow^{TM}.

Llevan más de una década desarrollándose métodos online para la determinación de elementos traza en el medio ambiente. Las matrices más comúnmente analizadas son las aguas medioambientales. Los primeros métodos online para pesticidas fueron desarrollados en 1998 [152, 153]. Tras ellos, otros muchos [154-160]. Otros analitos determinados en las aguas medioambientales mediante métodos online son herbicidas por [161], estrógenos por [149, 162, 163], detergentes [164], productos de cuidado personal [165], hidrocarburos aromáticos policíclicos [166], y otros disruptores endocrinos [167, 168]. Son también varios los trabajos que se pueden encontrar en la bibliografía, que emplean métodos de preparación de muestra, acoplados a técnicas cromatográficas para el análisis de fármacos en matrices no medioambientales, como en músculo de animales [169], o leche bovina [170]. No fue hasta el año 2004 que se desarrolló por primera vez un método online para fármacos en muestras de agua ambiental. En la Tabla 3 se recoge una revisión bibliográfica de métodos analíticos online para fármacos en aguas naturales, así como residuales y potables, por su relación directa con las primeras.

2.1.3 Análisis instrumental

Los avances en la etapa de pretratamiento han contribuído al rendimiento y fiabilidad del método. Pero ha sido las innovaciones tecnológicas en el análisis instrumental las que han hecho posible la detección de compuestos orgánicos traza. Así, el desarrollo del detector de captura de electrones (*Electron Capture Detector*, ECD) en GC a principios de los años '60, permitió descubrir que los pesticidas organoclorados y los policlorobifenilos (PCBs), se encontraban también en biota y en alimentos [108]. Fue por lo tanto, la GC, la primera técnica instrumental utilizada para la determinación de fármacos, y hasta los años '80, la mayoría de los métodos descritos en la literatura para el análisis de fármacos estaban

Tabla 3. Revisión bibliográfica de métodos analíticos online para fármacos en aguas naturales, residuales y potables

	Año publicación	País investigador	Nº compuestos / fármacos	Matrices1	Tamaño de muestra (mL)	Online sample preparation2	Sistema online	Sorbente	Técnica cromatográfica y detección3	Ref.
1	2011	España	8 / 4	SW, EWW	10	SPE	Precolumna en válvula de 6 puertos	Upchurch Scientific HXLPP	HILIC-ESI-MS	[142]
2	2010	Suiza	24 / 3	WW, SW	2 x 10	SPE	Precolumna en válvula de 6 puertos	Strata-X	LC-ESI-MS/MS	[171]
3	2010	España	11 / 11	SW, EWW	SW: 250; EWW: 100	SPE	Precolumna en válvula de 6 puertos	Upchurch Scientific HXLPP-WAX resin	LC-UV	[123]
4	2010	España	74 / 74	GW, SW, EWW, IWW	2.5	SPE	Spark Holland SymbiosisTM Pico	HySphere Resin GP	LC-MS/MS	[22]
5	2009	España	1 / 1	WW	5	Derivatización			GC-MS	[172]
6	2009	Estados Unidos	6 / 6	DW, SW, EWW, IWW	1	SPE	Spark Holland SymbiosisTMPharma	Waters Oasis HLB Prospekt	LC-MS/MS	[146]
7	2009	Canadá	14 / 6	DW, SW	10	SPE	Thermo Fisher Scientific EquanTM	Strata-X	LC-TOF	[52]
8	2009	Canadá	6 / 6	SW	1.5	ø			LC-MS/MS	[173]
9	2009	Canadá	5 / 5	SW, EWW, IWW	1	SPE	Thermo Fisher Scientific EquanTM	Strata-X	LC-ESI-MS/MS	[174]
10	2008	Argentina	8 / 8	SW	3	SPME		Polydimethylsiloxane/ divinylbenzene fibers	LC-UV	[139]
11	2008	Canadá	10 / 4	DW, SW, WW	1	SPE	Thermo Fisher Scientific EquanTM	Thermo Fisher Scientific Hypersil Gold C18	LC-ESI-MS/MS	[175]

12	2007	Canadá	6 / 6	WW	1	SPE	Thermo Fisher Scientific EquanTM	Thermo Fisher Scientific Hypersil Gold C18	LC-MS/MS	[176]
13	2007	Corea	14 / 14	WW	10	SPE	Spark Holland Prospekt	Waters Oasis HLB	LC-MS/MS	[177]
14	2006	España	16 / 16	GW, SW	9.8	SPE	Gilson 233XL	C-18	LC-MS/MS	[178]
15	2006	Irlanda	31 / 31	SW, DW	500	Columna de sílice monolítica de fase micro-invertida	Precolumna en válvula de 6 puertos	Merck Chromolith C18 monolithic silica	LC-UV-ESI-MS	[122]
16	2006	España	6 / 6	SW, WW	Inyección en continuo	SPE	Piston multi-jeringa	Waters Oasis HLB	LC-UV	[124]
17	2005	Suiza	28 / 10	SW	18	SPE	Precolumna en válvula de 6 puertos	Waters Oasis HLB	LC-MS/MS	[179]
18	2004	España	5 / 5	DW, GW, SW	0.025	cLC	Thermo Fisher Scientific EquanTM	Aqua C18	LC-MS/MS	[180]

[1]WW: *Waste Water*; IWW: *Influent Waste Water*; EWW: *Effluent Waste Water*; SW: *Superficial Water*; GW: *Groundwater*; DW: *Drinking Water*

[2]cLC: *Capillary-column-switching Liquid Chromatography*

[3]HILIC: *Hydrophylic Interaction Liquid Chromatography*; ESI: *ElectroSpray Ionization; TOF: Time Of Flight*

basados en la GC [108]. Así por ejemplo, sobre matrices biológicas, Roseboom y colaboradores [103] utilizaron GC con un detector UV y, Huang et al. [101] GC con un detector de ionización de llama (*Flame Ionization Detector*, FID) también acoplado a GC. Sobre matrices alimentarias, [104] utilizaron GC-ECD. Más tarde, el rápido desarrollo producido en el campo de la MS y MS/MS, la convirtieron en la técnica de detección por excelencia en el análisis medioambiental, reemplazando los detectorores habitualmente utilizados hasta el momento (UV, FID, ECD, etc.) [134]. Con la MS, la fiabilidad, selectividad y la sensibilidad mejoraron cuantitativamente, consiguiendo en ésta última límites de detección instrumental del orden de los picogramos [181]. Sin embargo, la GC no es una técnica que se pueda aplicar directamente para la determinación de compuestos polares, y la mayoría de los fármacos lo son. Por lo tanto, requiere una etapa de preparación de muestra más exhaustiva, añadiendo una etapa de derivatización tras el *cleanup*, para convertirlos en sustancias análogas menos polares, más volátiles y estables a elevadas temperaturas. Algunos ejemplos de métodos para el análisis de fármacos mediante GC-MS están descritos en [182-186]. Esta derivatización consume tiempo extra y presenta baja reproducibilidad [187]. Esto, junto con el importante desarrollo llevado a cabo sobre la HPLC, propició la aparición de métodos analíticos para fármacos basados en LC-UV, en el campo del desarrollo farmacéutico [98], sobre matrices biológicas [99, 100, 103], así como aguas ambientales [37, 139]. Y ya más tarde, la aparición de nuevas técnicas de ionización a presión atmosférica, tales como la ionización por electrospray (*ElectroSpray Ionization*, ESI) y la ionización química a presión atmosférica (*Atmospheric Pressure Chemical Ionization*, APCI), susceptibles de ser usadas como interfases para el acoplamiento LC-MS, propiciaron que desde mediados de los años '90, la LC-MS [188, 189] y sobre todo la LC-MS/MS [107, 108, 129, 134, 144, 190], se convirtiera en la técnica por excelencia para el análisis de fármacos en matrices ambientales [147]. Con el tiempo, la ESI se ha situado como la interfase más utilizada debido a su versatilidad. Otro beneficio que ofrece la LC acoplada a MS/MS es que, si los analitos no son isómeros, no precisa que éstos se separen completamente para poder detectarlos selectivamente [106]. No obstante, es siempre aconsejable conseguir una buena separación cromatográfica a fin de evitar o reducir los efectos del denominado efecto matriz, que típicamente resultan en la supresión o ensalzamiento anómalo de las señales correspondientes a los analitos. Por lo tanto, generalmente se utilizan columnas cromatográficas cortas, acortando el tiempo de análisis considerablemente. Las medidas más habituales de las columnas utilizadas para fármacos son, entre 10 y 25 cm de longitud, entre 2.1 y 4.6 mm de diámetro interno, y entre 1.7 y 5 µm de tamaño de partícula. La LC de fase invertida es ampliamente utilizada, siendo la columna C18 la preferida. Para fármacos muy polares, las columnas HILIC (*Hydrophilic Interaction Liquid Chromatography*) serían probablemente las más adecuadas, pero en los métodos multi-residuo, que analizan éstos junto con otros compuestos más apolares, se recurre habitualmente a la fase invertida.

Como fases móviles, acetonitrilo (ACN), MeOH o mezclas de ambos disolventes son normalmente usados, obteniéndose para el último caso, tiempos de retención más largos pero con mejor resolución de los analitos [191]. Para obtener una retención eficiente de los analitos en la columna y mejorar la sensibilidad de la detección por MS, se recomiendan, y de hecho se utilizan ampliamente, modificadores de fase móvil, soluciones reguladoras, ácidos y bases. Éstos deben ser volátiles y los más comunes son acetato amónico, formiato amónico, ácido fórmico y ácido acético. Concentraciones habituales varían entre 2 y 20 mM, ya que se ha observado que concentraciones más altas conllevan una reducción de la intensidad de señal [191].

Los fármacos se pueden clasificar en dos grupos según su mayor o menor tendencia a ionizarse en la interfase en modo positivo (*Positive Ionization*, PI) o negativo (*Negative Ionization*, NI). Debido a que las propiedades físico-químicas de los compuestos incluídos en cada grupo difieren considerablemente, normalmente se requieren diferentes fases móviles para cada modo de ionización. Generalmente, para fármacos ácidos, los cuales son determinados en modo NI, se prefieren fases móviles sin modificadores o si llevan, acetato amónico es el más comúnmente utilizado. Sin embargo, para modo PI, donde compuestos neutros y básicos son analizados, las fases móviles están modificadas a pH neutro o ácido [134]. A pesar de que APCI, en general proporciona menor efecto matriz que ESI, para los fármacos que son compuestos polares y en ocasiones pequeños, ESI es la interfase más versátil en cuanto a sensibiliadad. Así por ejemplo, la sensibilidad en reguladores de lípidos puede llegar a ser hasta diez veces mayor con ESI que con APCI [134, 192].

La Decisión Europea [193] indica criterios de identificación y confirmación en la determinación analítica de contaminantes en el medioambiente. Esta directiva fue originalmente definida para la determinación de contaminantes orgánicos en muestras alimentarias, y se ha expandido a otras matrices, como las medioambientales [106]. La decisión describe características a cumplir por los métodos. Para la confirmación de productos farmacéuticos, se requiere un mínimo de 4 puntos de identificación. Así, cuando se usa analizador de QqQ, se deben monitorizar dos transiciones entre el precursor y los iones producto, cuando se trabaja en modo SRM [106].

Una manera de aumentar la selectividad, y evitar falsos positivos es el uso de analizadores de masa como el TOF (*Time of Flight*) u Orbitrap, puesto que son capaces de proprocionar la masa exacta de los entes que detectan. Así por ejemplo, Stolker et al. [192] desarrolló métodos confirmatorios por LC acoplada a MS/MS, donde el primer analizador era un cuadrupolo (Q) y el segundo un TOF (LC-Q-TOF), de residuos farmacéuticos detectados previamente en aguas por LC-QqQ. Además, y por la misma razón, la LC-TOF se ha convertido en una herramienta importante en la identificación de compuestos farmacéuticos y sus productos de transformación en muestras medioambientales [134].

Los TOF son analizadores de masas muy rápidos, capaces de registrar eficientemente el espectro de masas completo con masa exacta a lo largo de todo un cromatograma. De esta manera registra, y por lo tanto, queda almacenada, una gran cantidad de información. Así, como si de una huella dactilar se tratase, el escáner completo de la composición de una muestra, quedará a disposición para futuras interpretaciones, si así se necesitase. Además, con el desarrollo tecnológico actual, se pueden encontrar analizadores TOF que proporcionan sensibilidades comparables con las de los QqQ [194]. La única desventaja, entonces, que desplaza a los TOF de su uso masivo en la determinación de niveles de concentración, es que proporcionan un rango de linealidad bajo, de dos o tres órdenes de magnitud, en general, en comparación con los QqQ que pueden llegar incluso a los cinco órdenes. El Orbitrap es un nuevo analizador comercializado por Thermo Fisher Scientific que ha revolucionado el sector, puesto que agrupa las bonanzas de ambos dos analizadores QqQ y TOF [195]Su fundamento difiere con respecto a cualquier otro analizador anterior y es capaz de proporcionar masa exacta, manteniendo un amplio rango de linealidad y alta sensibilidad. Esto le convierte en un analizador muy versátil. Ha sido rápidamente incorporado al servicio al análisis de fármacos en el medioambiente [196], tanto en estudios de cuantificación [197], como de identificación de desconocidos como productos de transformación [198-200].

2.1.3.1 Cromatografía de líquidos rápida de ultra elevada eficacia (Ultra High Performance Liquid Chromatography, UHPLC)

Ya se ha comentado anteriormente, que debido al creciente número de muestras que los laboratorios de estudios medioambientales tienen que procesar, la tendencia actual se dirige hacia el desarrollo de métodos de análisis de alto rendimiento, es decir, de corto tiempo de análisis por muestra, que proporcionen la capacidad de analizar la mayor cantidad de muestras en el menor tiempo posible, manteniendo o incluso mejorando la fiabilidad y demás características de los métodos predecesores. Como se ha dicho en el apartado 2.1.2, una de las etapas donde se ha realizado mayor desarrollo tecnológico en aras de la reducción del tiempo de análisis por muestra es la preparación de muestra. Otra etapa susceptible de ser acortada es la separación cromatográfica. Además, el acortamiento en esta fase no es excluyente del acoplamiento de la fase de pretratamiento a la cromatográfica.

La denominada cromatografía rápida, busca conseguir la misma o mejor resolución en la separación de los compuestos de interés en un menor intervalo de tiempo. Una manera de obtener tiempos de retención más cortos es trabajar a flujos de fase móvil mayores. Las columnas habitualmente utilizadas en la HPLC convencional, consisten en partículas compactas de diámetro entre 3 y 5 µm empaquetadas dentro de una estructura cilíndrica metálica rígida. El aumento del caudal de la fase móvil provoca el incremento de la presión

en el sistema, y si ésta supera los 350 bares se convierte en insoportable para los sistemas HPLC convencionales, y disminuyen la eficacia de la separación. Para superar esta limitación, durante la última década se han utilizado diferentes técnicas y tecnologías como las que se describen a continuación:

- Incremento de la temperatura de trabajo de la columna cromatográfica. Introduciendo la columna cromatográfica en un horno y aplicando una temperatura controlada, se consigue una disminución de la viscosidad de la fase móvil, y por lo tanto, de su resistencia al paso por la columna. Esto capacita la utilización de flujos de fase móvil mayores, a presiones dentro de los límites de la cromatografía convencional.

- Uso de otro tipo de columnas, como las columnas monolíticas o las denominadas "superficialmente porosas". Las primeras no están constituidas por partículas sino por un relleno de una sola pieza perforado por canales por los que la fase móvil puede discurrir ejerciendo una presión mucho menor que sobre las empaquetadas. En las "superficialmente porosas", el relleno de las columnas lo constituyen partículas empaquetadas con tamaño de partícula entorno a 3 µm, con la peculiaridad añadida de que constan de un núcleo compacto rodeado por una corteza porosa, que alivia parcialmente el aumento de presión que la fase móvil ejerce a su paso. Ambas opciones permiten el uso de flujos mayores, obteniendo presiones razonables dentro del contexto de la HPLC.

No obstante, el uso de las anteriores cromatografías rápidas no es tan común como la denominada cromatografía de líquidos de ultra alta eficacia (*Ultra High Performance Liquid Chromatography*, UHPLC) [201]. Consiste en el empleo de columnas empaquetadas de tamaño de partícula inferior a 2 µm. Con este planteamiento se mejora drásticamente el rendimiento de la HPLC convencional. La ecuación de van Deemter muestra que la eficacia de la separación cromatográfica está fuertemente influida por el tamaño de partícula. Las partículas pequeñas proporcionan un flujo más uniforme y una menor difusión del soluto a lo largo de la columna. Disminuyendo el tamaño de partícula del empaquetado, se reduce la altura del plato teórico, posibilitando el uso de columnas más cortas y ampliando el abanico de flujos a utilizar, sin sacrificar la eficacia. Por lo tanto, con flujos mayores o incluso similares, se obtienen tiempos de retención más cortos y picos más estrechos, manteniendo o mejorando la resolución en la separación y, por lo tanto, el método cromatográfico puede ser acortado. La recuperación de condiciones iniciales y acondicionmiento de la columna entre inyecciones es más rápida también, y así por ejemplo, comparando columnas UHPLC empaquetadas con partículas sub-2 mm, versus columnas de HPLC con tamaños de partícula de 3 mm, el tiempo de análisis debería acortarse al menos a un tercio [202]. Además, el hecho de que los analitos sean eluidos en picos más estrechos, puede propiciar mejoras en la sensibilidad. Otra ventaja de esta técnica es que, en la mayoría de los casos, el acortamiento en el tiempo de análisis cromatográfico, se traduce también en una reducción

del consumo de fase móvil. Como contrapartida, el empaquetamiento de partículas más pequeñas, deja menos espacio intersticial por la que circular la fase móvil, y así, la presión del sistema aumenta ostensiblemente, por encima de las capacidades de la HPLC convencional [201]. Por ejemplo, reduciendo el diámetro de partícula por tres, resulta en un aumento de la presión de columna de nueve veces para el mismo número de platos [202]. Por lo tanto, se hace necesaria el uso de instrumentación especial, como bombas capaces de soportar 15.000 psi, es decir, alrededor de 1.000 bares. Hasta el año 2000, las columnas disponibles no eran suficientemente estables para tales finalidades. Algunos investigadores como [203], empezaron a probar condiciones de UHPLC usando capilares de sílice fundida. Con ellas, el tiempo de análisis se redujo considerablemente, trabajando a velocidades lineales mayores y acortando la longitud de columna.

Las fases móviles utilizadas en los métodos UHPLC no difieren de las HPLC. De hecho, éstas pueden coincidir completamente si se traspasa un método HPLC a UHPLC. Así, MeOH y ACN son las fases orgánicas más habituales. Se prefiere quizás el ACN porque, aunque ambos presentan una resolución y reproducibilidad comparables, el ACN proporciona tiempos de retención más cortos y picos más simétricos. Además, el uso de ACN resulta en una reducción de dos en la presión del sistema en comparación con el MeOH debido a su baja viscosidad. El uso de modificadores, así como su naturaleza y concentración son idénticos a HPLC [204].

En general, el resto de etapas en un método analítico que incluye UHPLC, no tiene por qué diferir respecto a su equivalente en cromatografía convencional. La preparación de muestra puede ser traspasada directamente, como en [205], si bien, no existen hasta la fecha métodos online acoplados a UHPLC para el análisis de fármacos en muestras medioambientales. A pesar de que existen métodos para la determinación medioambiental de fármacos con detección UV [206-208], en general, la MS es en UHPLC como en HPLC, la técnica de detección más común. No obstante, debido a la rápida elución de los analitos en picos generalmente de anchura entre 2 y 6 segundos, los detectores deben ofrecer velocidades de adquisición elevadas para registrar un número razonable de puntos por pico, entre 12 y 15, para que la posterior cuantificación sea rigurosa. Los tres tipos de analizadores de masas más comunes son el QqQ, el TOF y el Orbitrap. Los QqQ de última generación son lo suficientemente potentes en términos de velocidad de adquisición, con pausas muy cortas entre ciclos, como para propiciar su acoplamiento con los sistemas UHPLC en métodos multi-residuo. Los antiguos, también, pero con limitación del número de transiciones registradas, en caso de SRM, y por lo tanto, del número de compuestos a analizar, o acotando el intervalo de escaneo de masas, en caso de trabajar en “Full Scan” [209]. No obstante, el analizador que es compatible sin problema con UHPLC es el TOF [210], ya que es capaz de registrar y almacenar los espectros de masas completos a velocidades de hasta 100 Hz [211]. Esto es debido a que es capaz de registrar todos los iones introducidos en el tubo de vuelo en cada pulso. En cualquier caso, para ambos analizadores, a mayor velocidad de adquisición, se disminuye el número de cuentas acumuladas para

generar el espectro, y por lo tanto, la sensibilidad empeora. El Orbitrap también presenta una velocidad de adquisición alta en su versión Exactive™ [204].

Agilent, con su 1200 series fue la primera casa comercial en sacar a la venta, en 2003, un sistema cromatográfico capaz de soportar presiones por encima de 400 bares. Al año siguiente Waters presentó su Acquity UPLC. Ambos sistemas permiten utilizar columnas cortas, entre 50 y 100 mm, 4.6 mm de diámetro interno, empaquetadas con partículas de tamaño de poro sub-2 µm (por ejemplo, las columnas Agilent RRHT de 1.8 µm, o las Acquity de 1.7 µm). Hoy en día la mayoría de los fabricantes del sector fabrican un sistema de altas presiones: Thermo Fisher Scientific tiene el Accela, Jasco el Xtrem-CC, Shimadzu el UFLC-XR, Hitache el LaChrom Ultra, etc. No obstante, hay que distinguir entre las capacidades de cada uno. Así por ejemplo, el Accela de Thermo es capaz de trabajar con presiones hasta 600 bares. Los Acquity de Water llegan a los 1.000 bares, y el nuevo modelo de Agilent 1290 Infinity, puede superar los 1.200 bares.

Revisando la literatura científica, la mayoría de la aplicaciones de la UHPLC al análisis medioambiental de fármacos, se centran en matrices acuosas, como agua de río [54, 206, 208, 212-216], algunos métodos para agua de mar [217, 218] y agua subterránea [219]. Las matrices sólidas como sedimentos, suelo o fango de EDAR han recibido mucha menos atención, y solo se han encontrado unos pocos métodos incluyendo tecnología UHPLC [220-223]. La duración del método en todos los casos, incluyendo las etapas de acondicionamiento y recuperación de condiciones iniciales, varió entre 2 [206] and 25 min [216], dependiendo del número de compuestos a analizar y del flujo de trabajo. Fruto del óptimo acoplamiento con los TOF, son muchos los estudios medioambientales de búsqueda y confirmación de derivados desconocidos de fármacos publicados [210, 221, 224-227].

En la publicación científica 1 del presente capítulo, se describe el desarrollo de un método UHPLC para 74 fármacos en matrices medioambientales acuosas, como las aguas subterráneas, superfiales de agua dulce, y residuales tanto de entrada como de salida a la EDAR.

2.1.3.2 Cromatografia quiral

Debido a que la mayoría de las propiedades físico-químicas de los enantiómeros son semejantes, los materiales de las columnas cromatográficas convencionales no son capaces de separarlos. Es decir, presentan idéntico comportamiento en ambientes no quirales. Por lo tanto, para separar enantiómeros, éstos deben ser introducidos en un ambiente quiral (método directo) o ponerlos en contacto con un reactivo quiral para convertirlos en diastereoisómeros, que ya presentan diferencias físico-químicas y, por lo tanto, se pueden separar mediante una técnica aquiral (método indirecto). Esta es la razón por la que la

aplicación directa de las columnas cromatográficas convencionales no es suficiente para separarlos.

Existen ejemplos de separación quiral (directa o indirecta) mediante GC, electroforesis capilar, y cromatografía de fluidos supercríticos. Pero la principal técnica empleada en los últimos 10-20 años, desarrollada fundamentalmente por la industria farmacéutica [228], ha sido la HPLC [229]**.** En ésta, como método indirecto, existe la posibilidad de derivatizar previamente, generando diastereoisómeros susceptibles de separación por cromatografía no quiral. No obstante, esto puede provocar la alteración del porcentaje presente de cada enantiómero. Como métodos directos, existe la cromatografía quiral, con la que se generan diastereoisómeros temporales insitu, ya sea añadiendo aditivos adecuados a la fase móvil o mediante el uso de fases estacionarias quirales. En el primer caso, puesto que se requiere su adicción en continuo, se emplean grandes cantidades de aditivos, y debido a que son necesarios de elevada pureza enantiómerica, habitualmente son caros. Además, presenta una cromatografía pobre con baja eficiencia y mala simetría de picos [229]. Por lo tanto, en general, la opción más extendida, es el uso de cromatografía quiral mediante columnas quirales. Las columnas quirales deben tener un relleno, que además de ser quiral, debe ser resistente a las condiciones habituales de temperatura, presión, disolventes y fuerza iónica de la HPLC [230]. Actualmente existen en torno a 170 fases estacionarias quirales disponibles comercialmente [231], capaces de llevar a cabo separación enantiomérica de prácticamente todo tipo de compuestos, actuando en diferentes modos cromatográficos, estos son, fase normal, fase invertida, orgánica polar e iónica polar. En general, las fases estacionarias quirales, se basan en biopolímeros (proteínas o polisacáridos), polímeros sintéticos, ciclodextrinas, antibióticos macrólidos, éteres de corona quiral, selectores tipo Pirkle, selectores de intercambio iónico quiral o selectores de intercambio de ligandos [229, 232]. No obstante, los más utilizados con fármacos son los polisacáridos y los glicopéptidos macrocíclicos, debido a su menor precio y al amplio abanico de compuestos que éstos rellenos resuelven. Columnas de este tipo, bajo los nombres Chirobiotic V y V2 (con vancomicina como fase estacionaria), T y T2 (con telcoplanina como fase estacionaria) y R (con ristoceitina como fase estacionaria), son comercializadas por la casa comercial Sigma-Aldrich, y Chiralcel y Chiralpak, por la casa Daicel.

En la literatura científica se pueden encontrar métodos de separación enantiomérica para una gran cantidad de fármacos mediante HPLC usando detectores UV. Como se ha comentado previamente, el uso de detectores más sensibles y selectivos como los MS, son imprescindibles para la determinación de fármacos en muestras medioambientales. Pero, la transferencia de los métodos quirales HPLC-UV a HPLC-MS no es inmediata, debido a que las fases móviles habitualmente usadas en los primeros, son incompatibles con las interfases de ionización de los instrumentos de MS. Así por ejemplo, las disoluciones amortiguadoras de fosfato no son lo suficientemente volátiles, o los disolventes de fase normal, como el hexano, no permiten la ionización en la interfase. Su uso, por lo tanto, conllevaría una disminución drástica en la sensibilidad, además de la contaminación del instrumento. Los

detectores de MS tienen también una limitación de flujos de trabajo mucho más acusada que los de UV [233]. Por lo tanto, en la mayoría de los casos, es necesario el desarrollo de nuevos métodos cromatográficos apropiados para MS. No obstante, esto se convierte en una tarea complicada, porque el uso de fases móviles compatibles con la MS, en muchos casos conduce a pérdidas de enantioselectividad. En cualquier caso, existen disponibles varios métodos de separación enantiomérica de fármacos en matrices como plasma, sangre y orina [234-239]. Pero no tantos en matrices medioambientales [141, 240-245]. Y además incluyen normalmente un pequeño número de compuestos cada vez.

2.1.4 Validación

Como con cualquier otro método, tras el desarrollo de uno para la determinación de fármacos en aguas medioambientales, se estima la bonanza de éste, mediante la validación de una serie de parámetros, que habitualmente son la precisión, la sensibilidad, el intervalo de linealidad, el efecto matriz y la exactitud.

La *precisión* nos da idea de la robusted del método, es decir, cuán repetibles son los resultados que éste genera. Para determinarla normalmente se recurre al cálculo de la repetibilidad a lo largo del tiempo, por ejemplo, comparando los resultados de muestras idénticas procesadas a lo largo de un mismo día y en días diferentes.

La *sensibilidad* es normalmente caracterizada por los límites de detección (*Limit of Detection*, LOD) y cuantificación (*Limit of Quantification*, LOQ) para cada analito incluido en el método y en cada matriz a aplicar. En general, los niveles de concentración encontrados en las aguas medioambientales y residuales varían entre los ng L^{-1} y los µg L^{-1}, dependiendo del compuesto y tipo de muestra [106]. Para la gran mayoría de los fármacos analizados mediante LC-QqQ trabajando en SRM, los LODs no superan los 50 ng L^{-1}, e incluso bajan por debajo de 1 ng L^{-1} para muchos compuestos, especialmente en aguas superficiales y subterráneas dulces, valores que se consideran suficientes para detectar y cuantificar con rigurosidad. La sensibilidad lograda con los métodos basados en GC-MS es tan buena o mejor, pero el tener que añadir una etapa de derivatización en los compuestos polares, la hacen una técnica menos interesante en análisis medioambiental de fármacos [134].

El *intervalo de linealidad* indica, el intervalo de concentraciones para el que la señal, aumenta proporcionalmente y de manera constante, con la concentración. Los instrumentos de QqQ generalmente proporcionan un intervalo de linealidad más amplio que los TOF. En los primeros, la linealidad supera generalmente los cuatro órdenes de magnitud, pudiéndose llegar incluso a cinco. Sin embargo, los TOF apenas cubren dos o tres [134]. De hecho, éste es uno de los principales problemas que los tiempo de vuelo presentan cuando se pretende su uso como herramientas de determinación cuantitativa [106]. Se desean intervalos de linealidad amplios en los órdenes de magnitud de trabajo,

para que incluso las concentraciones más elevadas de analito, estén dentro del intervalo de linealidad, y no tener, así, que recurrir a engorrosas prácticas como la dilución de la muestra, lo que complicaría el método, e influiría negativamente en su rapidez y fiabilidad.

El *efecto matriz*, consiste en el desfase producido en la intensidad de la señal como consecuencia de la presencia de componentes de la matriz en la muestra. En la GC-MS no es tan acusada, sin embargo, todas las interfases de ionización a presión atmosférica actualmente utilizadas en el acoplamiento LC-MS, son susceptibles a efecto matriz. No obstante, la ESI es más propensa a éste que el APCI o el APPI (*Atmospheric Pressure Photoionization*). En cualquier caso, con estas interfases, el efecto matriz es tanto más acusado cuanto más compleja es la matriz, especialmente si presenta un alto contenido en compuestos orgánicos disueltos, como el agua residual [208] o una alta salinidad, como el agua del mar [218]. Los componentes de la matriz compiten con los analitos de interés durante la ionización, resultando en una supresión (que pudiera incluso generar falsos negativos en el caso extremo de anulación de señal) o aumento anormal de la señal detectada para los analitos [108]. La cuantía de este efecto se calcula como el porcentaje de desvío de señal frente a una disolución en disolvente con la misma concentración de analitos, exenta, así, de los componentes de la matriz. Para el caso del análisis de muestras de aguas medioambientales o residuales, el disolvente consiste en agua ultra pura. Existen diversas estrategias que permiten reducir el efecto matriz [218, 246]. En primer lugar, deben procurarse extracciones selectivas de los analitos de interés durante la etapa de preparación de la muestra, realizar una etapa de purificación selectiva, o bien optimizar la separación cromatográfica. Sin embargo, éstas no son las soluciones más adecuadas en los métodos multi-residuo, donde su aplicación podría provocar pérdida de analitos, así como alargamiento del tiempo de análisis. Otras estrategias a plantear son el uso de métodos de calibración adecuados, como la adicción estándar, el patrón interno o la calibración externa en matriz [247-249]. Un desarrollo riguroso y fiable de la última opción se presenta imposible en este caso por la ausencia de blancos idénticos a las muestras medioambientales. La adicción estándar es un método fiable, pero por otro lado es tedioso y largo [248, 250]. Es por lo tanto, el calibrado con patrón interno la aproximación más eficaz, y de hecho, más común, para corregir el efecto matriz. Es una opción rápida. No obstante, su éxito depende en gran medida de la elección y asignación de los patrones internos. Éstos deben ser estructuras lo más análogas posibles a los analitos de interés, y que al mismo tiempo, no se encuentren presentes en la muestra. Su comportamiento físico-quimico debe ser similar. Además, como los componentes de la matriz también están sometidos a separación cromatográfica, el efecto matriz también depende del tiempo de retención, y así, los patrones internos deben eluirse lo más próximos posible a los analitos a los que corrigen. Es habitual, por lo tanto, que estos sean compuestos pertenecientes a la misma familia, o incluso los mismos analitos marcados isotópicamente, lo que supone el mejor de los escenarios. Por lo tanto, en los métodos multi-residuo habitualmente se requiere más de un patrón interno para proceder con una corrección rigurosa de todos los

analitos [134]. De hecho, la situación óptima es lo que se conoce como dilución isotópica [251], y consiste en el uso del correspondiente analito marcado isotópicamente para cada residuo incluido en el método. Los inconvenientes a esta aproximación en los métodos multi-residuos son la falta de disponibilidad comercial de isótopos de algunos fármacos, y sobre todo, el elevado coste asociado. Otras estrategias menos comunes son la disminución del caudal que se envía al detector de masas o la dilución de los extractos de muestra como en [130].

No obstante, por otro lado, como afirman varios autores [215, 226, 252], aplicados sobre una misma muestra, el acoplamiento UHPLC-MS proporciona menos efecto matriz que el HPLC-MS. Esto es debido a que la UHPLC resuelve mejor y genera picos más estrechos que la HPLC. Por lo tanto, la probabilidad de solapamiento entre los picos de los analitos y de la matriz es menor [252].

La *exactitud* es la determinación del error sesgo del método. Esto habitualmente se calcula mediante los porcentajes de recuperación absolutos de disoluciones de concentración conocida, procesadas de manera idéntica a como si fuesen muestras problema. Para corregir este error, la opción más recomendada es de nuevo, el uso de dilución isotópica, es decir, de derivados isotópicos de todos y cada uno de los fármacos problema incluídos en el método. Estos compuestos deben ser añadidos en concentración conocida al inicio del proceso analítico. Así, comparando la concentración detectada al final del mismo con la añadida al inicio, se pueden preveer y corregir las variaciones en la señal que se habrán producido en el analito al que corrige. Si no se cuentan con derivados isotópicos para todos los residuos, se pueden asignar análogos estructurales, pero en ese caso, la corrección no es tan fiable, y en ocasiones solo actuarán de chivatos de algunas, pero no todas, la etapas del proceso analítico (ejemplo, solo de la SPE). Otra opción, es el cálculo de la exactitud del método. Para esto se dopan a concentración conocida las muestras problema, y posteriormente se procesan de manera paralela con el método analítico junto con las muestras problema sin dopar.

Las principales fuentes de falta de exactitud en el método son debidas al anteriormente descrito efecto matriz y la no idealidad en la extracción de los analitos durante la preparación de muestra.

2.2 Presentación de resultados

En el presente capítulo se presentan el desarrollo, optimización y validación de 4 métodos de análisis de fármacos y derivados en aguas medioambientales continentales, tanto superficiales como subterráneas, así como en aguas residuales. Estos métodos han sido además probados en muestras reales. Toda esta información está contenida en 4 artículos de divulgación científica:

***Método 1*:**

Publicación científica 1: “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)” por Rebeca López-Serna, Mira Petrović, Damià Barceló en “Chemosphere”

***Método 2*:**

Publicación científica 2: “Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry” por Rebeca López-Serna, Sandra Pérez, Antoni Ginebreda, Mira Petrović, Damià Barceló en “Talanta”

***Método 3*:**

Publicación científica 3: “Direct analysis of pharmaceuticals, their metabolites and transformation products in environmental waters using on-line TurboFlowTM chromatography-liquid chromatography-tandem mass spectrometry” por Rebeca López-Serna, Mira Petrović, Damià Barceló en “Journal of Chromatography”

***Método 4*:**

***Publicación científica 4*:** “Multi-residue enantiomeric analysis of pharmaceuticals and their active metabolites in the Guadalquivir River basin (South Spain) by chiral liquid chromatography coupled with tandem mass spectrometry” por Rebeca López-Serna, Barbara Kasprzyk-Hordern, Mira Petrović, Damià Barceló en “Analytical Bioanalytical Chemistry”

Al final del capítulo se discuten y comparan las características principales de cada método.

Publicación científica #1

“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)”

por:

Rebeca López-Serna, Mira Petrović, Damià Barceló

en “Chemosphere”

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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)

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ABSTRACT

This work describes the development, optimization and validation of an analytical method for the simultaneous detection and identification of 74 pharmaceutically active compounds (PhACs), from various therapeutic groups, in both environmental (ground and surface water) and wastewaters (WW). The method is based on the simultaneous extraction of all target compounds by solid phase extraction (SPE), using a hydrophilic–lipophilic balanced polymer followed by ultra high performance liquid chromatography (UHPLC) tandem mass spectrometry (MS/MS). Two selected reaction monitoring (SRM) transitions have been monitored per compound in order to fulfil the EC guidelines, as well as to ensure an accurate identification of target compounds in the samples. Quantification is performed by internal standard approach, applying 24 specific isotopically labeled compounds. The main advantages of the developed method, besides the selectivity and reliability of the results, is its high throughput. All compounds are extracted in a single step and the instrumental analysis lasts 5 min (NI mode) + 8 min (PI mode), allowing fast throughput of samples. The limits of detection range from 0.01 to 50 ng L^{-1}, depending on the matrix, for most of the compounds. Finally, the method developed has been applied to the analysis of pharmaceuticals in the Ebro river basin (NE Spain).

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1. Introduction

Although pharmaceutically active compounds (PhACs) are not new contaminants, the information concerning their concentration and environmental fate has only been reported in the last years (Halling-Sørensen et al., 1998; Ternes, 1998; Kümmerer, 2001; Kolpin et al., 2002; Thiele-Bruhn, 2003; Moldovan, 2006; Sarmah et al., 2006; Duong et al., 2008). Commonly used prescription and over-the-counter pharmaceuticals are typically found in surface and groundwater samples at concentrations lower than 1 μg L^{-1} (Katz and Griffin, 2008). These concentrations are generally considered too low to pose an acute risk for humans; however, it is still unknown whether other receptors in non-target organisms, like aquatic organisms, are sensitive to individual pharmaceutical residues, or their mixtures. Studies have shown that combinations of pharmaceutical compounds exert a much stronger toxic effect than could be expected from the weak toxic effects related to exposure to each compound individually (Cleuvers, 2003; Carlsson et al., 2006; Katz and Griffin, 2008). Moreover, the consumption of pharmaceuticals is steadily increasing all around the world; therefore the situation regarding their occurrence and levels concentration in the environment is expected to get worse (Heberer, 2002; Heberer and Feldmann, 2005; Reemtsma et al., 2006; Snyder et al., 2007; Chefetz et al., 2008; Katz and Griffin, 2008).

The prerequisite for proper monitoring and risk evaluation is availability of sensitive multi-residue analytical method for the determination of pharmaceutical compounds in complex environmental matrices. The detection of pharmaceuticals at the level of ng L^{-1} has been made feasible by the advances in mass spectrometry (Calisto and Esteves, 2009; Kümmerer, 2009a,b). At the same time, the extraction techniques have become more simple, fast and inexpensive, providing the enrichment of analytes of interest from complex matrices such as wastewater or sewage sludge. However, the growing number of samples to be analyzed in laboratories carrying out monitoring studies requires employment of high-throughput methods. Because of these reasons, great effort is going into the development of fast and, at the same time, reliable multicomponent methods capable of analyzing simultaneously

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several tens of compounds belonging to different therapeutical groups. One of the options, increasingly being used in environmental analysis, is ultra-high performance liquid chromatography (UHPLC) that employs columns packed with sub-2 μm particles, which enable elution of sample components in much narrower, more concentrated bands, resulting in better chromatographic resolution and increased peak capacity.

This paper describes development, optimization and application of a sensitive and fast method for the determination of pharmaceuticals using UHPLC–MS/MS (triple quadrupole). In comparison to several other methods based on UHPLC–MS/MS for the analysis of pharmaceuticals in water (Batt et al., 2008; Kasprzyk-Hordern et al., 2008; Van De Steene and Lambert, 2008; Huerta-Fontela et al., 2010; Morasch et al., 2010; Sui et al., 2010; Wu et al., 2010) this method offers improvements in terms of number of compounds monitored (74 PhACs), shorter chromatographic runs, more reliable quantification due to application of larger number of isotopically labeled internal standards (IS) and applicability to different aqueous matrices (groundwater, surface water, wastewater).

The pharmaceuticals included in this method belong to different families and were chosen because of their high consumption in Spain, previously reported occurrence in the environment, reported low elimination in wastewater treatment plants (WWTP) and ecotoxicological relevance. The method has been proved valid for environmental waters (ground (GW) and surface water (SW)) and sewage water (WWTP effluent (WWE) and influent (WWI)).

2. Material and methods

2.1. Chemicals

All chemicals used are listed in the Supplementary material. Both individual stock standard and isotopically labeled internal standard solutions were prepared on a weight basis in methanol (MeOH), except fluoroquinolones which were dissolved in a water:methanol mixture (1:1) that contained 0.2% v/v hydrochloric acid. Special precautions have to be taken into account for tetracycline antibiotics, which have to be stored in the dark in order to avoid their exposure to the light, since it has been demonstrated that they are liable to photodegradation (Eichhorn and Aga, 2004). Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of substances was renewed every three months.

A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in methanol–water (25:75, v/v). A separate mixture of isotopically labeled internal standards, used for internal calibration, was prepared also in methanol–water (25:75, v/v).

2.2. Sample pre-treatment

The same method was used for groundwater, river water, WWTP influent and effluent. Amber glass bottles were used for sample collection. Water samples were filtered through 1 μm glass fiber filters from Whatman (Fairfield, Connecticut, USA) followed by 0.45 μm nylon membrane filters from Teknokroma (Barcelona, Spain). Na_2EDTA (0.1% (m/v)) was added to all samples after filtration. The addition of this chelating agent improves the extraction recovery of some antibiotics, (Zhu et al., 2001; Yang et al., 2004, 2005; Batt et al., 2006; Hernández et al., 2007; Gros et al., 2009). For the pre-concentration of water samples, a Baker vacuum system (J.T. Baker, The Netherlands) was used. A volume of 500 mL of surface and ground waters, 200 mL of WWTP effluent and 100 mL of WWTP influent were loaded onto Oasis HLB cartridges (60 mg, 3 mL) from Waters Corporation (Milford, Massachusetts, USA), at a flow rate of approximately 5 mL min^{-1}. After sample pre-concentration, cartridges were rinsed with 5 mL of HPLC grade water and were dried under vacuum for 15–20 min, to remove excess of water. If not eluted immediately, the cartridges were frozen and kept at −20 °C. Nevertheless, the elution was typically performed within 2 days in all cases. Elution consisted of 2 consecutive volumes of 4 mL of MeOH each. Extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted with 0.5 mL of methanol–water (25:75, v/v).

Afterward, 75 μL of a 0.5 ng μL^{-1} standard mixture containing 18 internal standards for the analysis in positive ion (PI) mode and 6 internal standards for the analysis in negative ion (NI) mode (see Table 1), were added in the extract for internal standard calibration. Addition of isotopically labeled internal standards in the final extract compensate for possible matrix effect (ion suppression or enhancement), but does not compensate for any of the possible procedural losses (extraction, evaporation). In order to check for the recovery efficiency the extraction of spiked samples (same matrix) was regularly performed before each set of real samples.

Finally, all the extracts which presented turbidity (most of cases, extracts of WWTP influents) were filtered through 0.22 μm PTFE filters (Membrane solutions, USA) 13 mm of diameter in order to avoid chromatographic problems. PTFE is known to be very inert with all type of polarities. In fact, no significant differences were observed in preliminary trials with and without filtering extracts coming from spiked solutions. In any case, any loss of target compounds due to adsorption on precipitate or adsorption on PTFE filters, was corrected by the internal standards present in the extract.

2.3. UHPLC–MS/MS analysis

The chromatographic separation was performed using an Acquity UHPLC and a reversed-phase column BEHC18 (2.1 mm × 100 mm, particle size 1.7 μm) both from Waters Corporation (Milford, Massachusetts, USA). For MS/MS analysis, Acquity UHPLC was connected in series with an Acquity TQD, a triple quadrupole MS equipped with a ZSpray source (Waters Corporation (Milford, Massachusetts, USA)).

Compounds analyzed in the PI mode were eluted with a mobile phase consisting of (A) acetonitrile (ACN) and (B) 0.1% aqueous formic acid. The elution started at 10% A and then was linearly increased to 75% A in 6.2 min, further increased to 100% A in 0.35 min and kept isocratic for 0.5 min. The total time of chromatographic analysis (including re-equilibration) was 8 min. Compounds analyzed under NI conditions were eluted by (A) acetonitrile:methanol (1:1, v/v) and (B) 10 mM ammonium acetate. The elution gradient was linearly increased from 20% A to 80% A in 1.3 min, then increased to 90% A in 0.7 min and kept isocratic for 2 min. Total run time, including the conditioning of the column to the initial conditions was 5 min. In both modes, the flow rate was 0.4 mL min^{-1} and the injection volume 20 μL. Column was thermostated at 30 °C and samples were preserved in the injection station at 15 °C.

For quantitative analysis a method of electrospray ionization tandem MS (ESI–MS/MS) was developed. For most of compounds two selected reaction monitoring (SRM) transitions between the precursor ion and two most abundant fragment ions were monitored (full list of SRMs and instrumental conditions are given in Table 1). This allows accomplishment of the requirements set by the European Union (EU) regulations (EU Commission Decision 2002/657/EC) related to identification and confirmation of contaminants analyzed by LC–MS/MS. Just for ibuprofen and ketoprofen, due to their poor fragmentation, only one transition was monitored. One transition was also monitored for the isotopically labeled standards.

Table 1
Target compounds, optimized MS/MS parameters, corresponding internal standards and MLD in HPLC grade water.

Compounds	No.	Precursor ion (m/z)	SRM 1	CV–CE–Dwell time	SRM 2	CV–CE–Dwell time	Rt (min)	SRM1/ SRM2	Corresponding internal standard	MLD in HPLC grade water (ng L^{-1})
Salicylic acid	1	137.10 $[M-H]^-$	93.03	25–10–0.013	65.04	25–25–0.013	1.20	13.14	*Hydrochlorothiazide-d2*	1.54
Hydrochlorothiazide	2	296.12 $[M-H]^-$	205.03	30–20–0.013	268.97	30–15–0.013	1.42	1.06	*Hydrochlorothiazide-d2*	2.40
Hydrochlorothiazide-d2 (*IS*)	–	298.11 $[M-H]^-$	269.97	30–15–0.013	–	–	1.42	–		
Furosemide	3	329.18 $[M-H]^-$	205.08	35–20–0.013	285.08	35–15–0.013	1.98	1.07	*Ibuprofen-d3*	1.17
Clofibric acid	4	213.13 $[M-H]^-$	127.00	20–15–0.013	85.00	20–10–0.013	2.04	4.15	*Clofibric acid-d4*	0.11
Clofibric acid-d4 (*IS*)	–	217.27 $[M-H]^-$	131.09	20–15–0.013	–	–	2.04	–		
Pravastatin	5	423.45 $[M-H]^-$	101.13	30–25–0.013	321.39	30–15–0.013	2.11	1.15	*Naproxen-d3*	1.62
Naproxen	6	229.22 $[M-H]^-$	170.13	15–15–0.013	185.14	15–5–0.013	2.16	1.30	*Naproxen-d3*	0.37
Naproxen-d3 (*IS*)	–	232.30 $[M-H]^-$	173.19	5–15–0.013	–	–	2.16	–		
Ketoprofen	7	253.26 $[M-H]^-$	209.16	15–5–0.013	–	–	2.17	–	*Naproxen-d3*	1.20
Chloramphenicol	8	321.17 $[M-H]^-$	152.00	25–15–0.013	257.07	25–10–0.013	2.17	1.89	*Ibuprofen-d3*	0.05
Phenobarbital	9	231.17 $[M-H]^-$	188.23	20–10–0.013	85.15	20–10–0.013	2.18	2.49	*Phenobarbital-d5*	1.30
Phenobarbital-d5 (*IS*)	–	236.25 $[M-H]^-$	192.99	23–10–0.013	–	–	2.18	–		
Bezafibrate	10	360.22 $[M-H]^-$	274.12	25–15–0.013	154.01	25–25–0.013	2.24	2.55	*Clofibric acid-d4*	0.13
Butalbital	11	223.20 $[M-H]^-$	180.14	20–10–0.013	85.00	20–10–0.013	2.33	4.47	*Phenobarbital-d5*	0.72
Pentobarbital	12	225.23 $[M-H]^-$	182.22	25–10–0.013	85.06	25–10–0.013	2.46	16.58	*Phenobarbital-d5*	0.55
Diclofenac	13	294.16 $[M-H]^-$	250.04	15–10–0.013	214.11	15–20–0.013	2.47	28.52	*Diclofenac-d4*	0.30
Diclofenac-d4 (*IS*)	–	298.18 $[M-H]^-$	254.07	15–10–0.013	–	–	2.47	–		
Indomethacine	14	356.20 $[M-H]^-$	312.18	15–10–0.013	297.14	15–20–0.013	2.52	2.93	*Ibuprofen-d3*	0.44
Ibuprofen	15	205.22 $[M-H]^-$	161.15	15–5–0.013	–	–	2.57	–	*Ibuprofen-d3*	0.50
Ibuprofen-d3 (*IS*)	–	208.23 $[M-H]^-$	164.16	15–5–0.013	–	–	2.57	–		
Mefenamic acid	16	240.24 $[M-H]^-$	196.18	30–15–0.013	192.11	30–25–0.013	2.60	19.09	*Ibuprofen-d3*	0.18
Gemfibrozil	17	249.27 $[M-H]^-$	121.09	20–10–0.013	127.06	20–10–0.013	3.01	11.18	*Ibuprofen-d3*	0.07
Compounds analyzed by PI mode										
Salbutamol	18	240.17 $[M+H]^+$	147.99	15–20–0.013	222.07	15–10–0.013	1.02	1.58	*Albuterol-d3*	0.02
Albuterol-d3 (*IS*)	–	243.21 $[M+H]^+$	151.02	20–20–0.013	–	–	1.02	–		
Famotidine	19	338.10 $[M+H]^+$	188.89	15–20–0.013	259.04	15–10–0.013	1.06	1.37	*Cimetidine-d3*	0.04
Atenolol	20	267.23 $[M+H]^+$	144.98	25–25–0.013	74.00	25–25–0.013	1.07	1.11	*Atenolol-d7*	0.03
Atenolol-d7 (*IS*)	–	274.31 $[M+H]^+$	79.04	25–25–0.013	–	–	1.07	–		
Cimetidine	21	253.20 $[M+H]^+$	94.99	15–25–0.013	159.00	15–15–0.013	1.10	1.16	*Cimetidine-d3*	0.03
Cimetidine-d3 (*IS*)	–	256.20 $[M+H]^+$	162.00	15–15–0.013	–	–	1.10	–		
Sotalol	22	273.23 $[M+H]^+$	255.03	15–10–0.012	213.06	15–20–0.012	1.17	1.74	*Atenolol-d7*	0.19
Ranitidine	23	315.20 $[M+H]^+$	175.98	20–15–0.012	130.03	20–25–0.012	1.17	1.42	*Ranitidine-d6*	0.03
Ranitidine-d6 (*IS*)	–	321.27 $[M+H]^+$	176.09	20–15–0.012	–	–	1.17	–		
Acetaminophen	24	150.10 $[M+H]^+$	109.99	20–15–0.012	65.00	20–30–0.012	1.28	6.14	*Acetaminophen-d4*	2.46
Acetaminophen-d4 (*IS*)	–	156.13 $[M+H]^+$	114.02	30–15–0.012	–	–	1.28	–		
Metronidazole	25	172.08 $[M+H]^+$	128.00	15–15–0.012	82.03	15–25–0.012	1.33	1.62	*Ranitidine-d6*	0.06
Codeine	26	300.25 $[M+H]^+$	215.06	40–25–0.012	165.01	40–40–0.012	1.49	1.07	*Codeine-d3*	0.12
Codeine-d3 (*IS*)	–	303.28 $[M+H]^+$	164.97	40–40–0.012	–	–	1.49	–		
Sulfadiazine	27	251.08 $[M+H]^+$	155.93	20–15–0.012	91.99	20–25–0.012	1.60	1.61	*Sulfamethoxazole-d4*	0.12
Lisinopril	28	406.34 $[M+H]^+$	84.02	25–25–0.012	246.08	25–25–0.012	1.66	13.38	*Atenolol-d7*	0.10
Nadolol	29	310.00 $[M+H]^+$	201.00	30–25–0.011	254.00	30–25–0.011	1.77	1.66	*Atenolol-d7*	0.04
Trimethoprim	30	291.21 $[M+H]^+$	230.08	30–25–0.010	123.03	30–25–0.010	1.90	1.23	*Carbamazepine-d10*	0.03
Pindolol	31	249.00 $[M+H]^+$	116.00	25–20–0.010	144.00	25–25–0.010	1.90	5.06	*Atenolol-d7*	0.01
Norfloxacin	32	320.08 $[M+H]^+$	276.26	30–15–0.010	301.94	30–20–0.010	1.96	1.67	*Ofloxacin-d8*	0.70
Ofloxacin	33	362.15 $[M+H]^+$	318.18	30–20–0.010	261.11	30–30–0.010	1.96	1.28	*Ofloxacin-d8*	0.09
Ofloxacin-d8 (*IS*)	–	370.24 $[M+H]^+$	326.04	20–20–0.010	–	–	1.96	–		
Oxytetracycline	34	461.06 $[M+H]^+$	426.64	25–20–0.010	442.62	25–15–0.010	2.00	1.04	*Demeclocycline*	0.62
Ciprofloxacin	35	332.09 $[M+H]^+$	314.14	40–20–0.010	230.99	40–40–0.010	2.02	1.76	*Ofloxacin-d8*	8.98
Fluoxetine	36	310.15 $[M+H]^+$	148.18	20–10–0.011	91.17	20–50–0.011	2.02	2.10	*Fluoxetine-d5*	1.39
Fluoxetine-d5 (*IS*)	–	315.15 $[M+H]^+$	153.16	20–10–0.011	–	–	2.02	–		
Danofloxacin	37	358.14 $[M+H]^+$	82.02	35–40–0.010	340.23	35–25–0.010	2.09	2.22	*Ofloxacin-d8*	0.49
Sulfamethazine	38	279.15 $[M+H]^+$	186.03	20–15–0.009	155.86	20–20–0.009	2.15	1.48	*Sulfamethoxazole-d4*	0.13
Enrofloxacin	39	360.21 $[M+H]^+$	315.87	35–20–0.009	342.41	35–20–0.009	2.17	19.48	*Ofloxacin-d8*	0.04
Tetracycline	40	445.19 $[M+H]^+$	410.03	25–20–0.009	154.10	25–25–0.009	2.19	1.18	*Demeclocycline*	0.41
Phenazone	41	189.10 $[M+H]^+$	76.93	25–35–0.009	56.04	25–25–0.009	2.25	1.13	*Phenazone-d3*	0.77
Phenazone-d3 (*IS*)	–	192.14 $[M+H]^+$	59.05	30–25–0.009	–	–	2.25	–		
Timolol	42	317.23 $[M+H]^+$	261.11	20–15–0.009	244.16	20–20–0.009	2.29	2.88	*Atenolol-d7*	0.01
Metoprolol	43	268.27 $[M+H]^+$	74.00	20–20–0.009	116.09	20–20–0.009	2.43	1.23	*Atenolol-d7*	0.06
Clenbuterol	44	277.14 $[M+H]^+$	202.94	15–15–0.009	259.07	15–10–0.009	2.45	1.83	*Atenolol-d7*	0.04
Demeclocycline (*IS*)	–	465.23 $[M+H]^+$	448.07	35–15–0.009	–	–	2.45	–		
Spiramycin	45	843.57 $[M+H]^+$	174.34	50–40–0.009	142.43	50–35–0.009	2.60	4.64	*Azithromycin-d3*	1.04
Azithromycin	46	749.00 $[M+H]^+$	158.00	40–35–0.009	591.00	40–35–0.009	2.65	2.18	*Azithromycin-d3*	0.36
Azithromycin-d3 (*IS*)	–	752.75 $[M+H]^+$	116.05	40–50–0.009	–	–	2.65	–		
Chlortetracycline	47	479.09 $[M+H]^+$	154.07	30–30–0.009	461.99	30–20–0.009	2.78	1.72	*Demeclocycline*	1.14
Sulfamethoxazole	48	254.12 $[M+H]^+$	155.93	20–15–0.009	92.03	20–25–0.009	2.87	3.79	*Sulfamethoxazole-d4*	0.18
Sulfamethoxazole-d4 (*IS*)	–	258.16 $[M+H]^+$	95.98	20–30–0.009	–	–	2.87	–		
Carazolol	49	299.25 $[M+H]^+$	115.95	25–20–0.009	221.93	25–20–0.009	2.95	3.45	*Atenolol-d7*	0.03
Doxycycline	50	445.13 $[M+H]^+$	427.77	30–20–0.009	154.01	30–30–0.009	2.96	15.73	*Demeclocycline*	0.75
Tilmicosin	51	869.74 $[M+H]^+$	173.71	50–45–0.009	696.53	50–40–0.009	3.08	2.03	*Azithromycin-d3*	0.95
Enalapril	52	377.36 $[M+H]^+$	234.04	25–20–0.009	117.02	25–35–0.009	3.23	2.20	*Enalapril-d5*	0.09
Enalapril-d5 (*IS*)	–	382.32 $[M+H]^+$	239.08	20–20–0.009	–	–	3.23	–		
Propranolol	53	260.28 $[M+H]^+$	116.08	15–15–0.010	74.02	15–20–0.010	3.28	3.05	*Atenolol-d7*	0.02

Table 1 (*continued*)

Compounds	No.	Precursor ion (m/z)	SRM 1	CV–CE–Dwell time	SRM 2	CV–CE–Dwell time	Rt (min)	SRM1/ SRM2	Corresponding internal standard	MLD in HPLC grade water (ng L^{-1})
Betaxolol	54	308.32 $[M+H]^+$	72.00	30–25–0.010	116.02	30–20–0.010	3.40	1.27	*Atenolol-d7*	0.01
Erithromycin	55	734.58 $[M+H]^+$	157.96	35–30–0.011	82.99	35–50–0.011	3.51	2.62	*Erythromycin-13C-d3*	1.57
Erythromycin-13C-d3 (*IS*)	–	738.75 $[M+H]^+$	162.04	15–30–0.011	–	–	3.51	–		
Tylosin A	56	916.71 $[M+H]^+$	174.19	45–35–0.011	101.03	45–50–0.011	3.66	3.03	*Azithromycin-d3*	0.14
Carbamazepine	57	237.14 $[M+H]^+$	194.07	27–20–0.011	165.01	27–40–0.011	3.83	35.29	*Carbamazepine-d10*	0.06
Carbamazepine-d10 (*IS*)	–	247.17 $[M+H]^+$	204.05	25–20–0.011	–	–	3.83	–		
Paroxetine	58	330.23 $[M+H]^+$	69.99	30–30–0.011	192.07	30–20–0.011	3.87	2.36	*Carbamazepine-d10*	0.03
Flumequine	59	262.12 $[M+H]^+$	244.07	25–15–0.013	201.91	25–30–0.013	3.98	1.92	*Carbamazepine-d10*	0.51
Propyphenazone	60	231.18 $[M+H]^+$	56.01	30–30–0.013	189.03	30–20–0.013	4.04	1.55	*Phenazone-d3*	0.03
Clarithromycin	61	748.71 $[M+H]^+$	158.00	20–25–0.013	83.01	20–50–0.013	4.13	1.81	*Azithromycin-d3*	0.05
Enoxacin	62	321.13 $[M+H]^+$	303.01	35–20–0.014	232.16	35–35–0.014	4.20	7.65	*Ofloxacin-d8*	4.44
Lorazepam	63	321.07 $[M+H]^+$	302.94	25–15–0.014	274.84	25–20–0.014	4.20	1.34	*Diazepam-d5*	1.42
Roxithromycin	64	837.80 $[M+H]^+$	158.12	20–35–0.013	679.76	20–20–0.013	4.21	2.48	*Azithromycin-d3*	0.03
Josamycin	65	828.76 $[M+H]^+$	109.05	40–45–0.016	174.01	40–30–0.016	4.44	1.24	*Azithromycin-d3*	0.02
Loratadine	66	383.24 $[M+H]^+$	337.11	35–25–0.016	267.15	35–35–0.016	4.49	2.23	*Cimetidine-d3*	0.04
Nifuroxazide	67	276.11 $[M+H]^+$	120.98	35–20–0.017	92.97	35–40–0.017	4.79	12.32	*Carbamazepine-d10*	1.15
Diazepam	68	285.18 $[M+H]^+$	153.96	40–25–0.017	193.04	40–30–0.017	4.93	1.17	*Diazepam-d5*	0.16
Diazepam-d5 (*IS*)	–	290.13 $[M+H]^+$	198.08	40–30–0.017	–	–	4.93	–		
Glibenclamide	69	494.00 $[M+H]^+$	369.00	25–15–0.027	169.00	25–40–0.027	5.61	1.05	*Glyburide-d3*	0.19
Glyburide-d3 (*IS*)	–	497.00 $[M+H]^+$	372.00	25–15–0.027	–	–	5.61	–		
Atorvastatin	70	559.37 $[M+H]^+$	440.02	25–20–0.027	250.03	25–40–0.027	5.61	1.48	*Carbamazepine-d10*	0.18
Tamoxifen	71	372.36 $[M+H]^+$	72.03	30–25–0.027	129.00	30–25–0.027	5.67	34.47	*Carbamazepine-d10*	0.01
Phenylbutazone	72	309.24 $[M+H]^+$	120.02	25–20–0.032	160.03	25–20–0.032	5.95	2.04	*Phenazone-d3*	0.49
Mevastatin	73	391.35 $[M+H]^+$	159.03	15–27–0.053	185.10	15–25–0.053	6.41	1.00	*Carbamazepine-d10*	1.01
Fenofibrate	74	361.00 $[M+H]^+$	233.00	25–20–0.143	139.00	25–25–0.143	7.46	1.10	*Fenofibrate-d6*	0.17
Fenofibrate-d6 (*IS*)	–	367.24 $[M+H]^+$	138.93	30–30–0.143	–	–	7.46	–		

3. Results and discussion

3.1. UPLC–MS/MS analysis

3.1.1. Optimization of chromatographic separation

To optimize the chromatographic separation, a serial of preliminary experiments were performed, testing different mobile phases like H_2O, 0.1% formic acid (FA), 10 mM ammonium formate and 10 mM ammonium acetate as aqueous mobile phases, while ACN, 0.1% FA in ACN, MeOH/ACN and 10 mM ammonium acetate in MeOH/ACN as organic phases.

The optimal separation, best peak shape and higher intensity (in terms of intensity of the analyte signal, measured in number of counts per second) of the 57 compounds (and 18 internal standards) analyzed under PI was achieved by using ACN as organic phase and water with 0.1% FA as the aqueous phase.

For the analysis of 23 substances (17 compounds and 6 internal standards) under NI mode, the optimum separation was obtained with a mixture of ACN/MeOH (1:1, v/v) as organic phase and 10 mM ammonium acetate in water. Although the addition of that modifier to the aqueous phase decreases slightly the signal, the peak shape improves considerably. For both modes, several gradient designs (different flow rates and slopes) were tested in order to get the maximum separation of every compound in a shorter run time. The optimized gradients (see Section 2.3.) resulted in 5 min chromatographic runs for NI mode and 8 min for PI mode. Column temperature was also optimized and 30 °C, controlled by a thermostat, was chosen as the temperature giving the best chromatographic performance (shorter and stable retention times, good peak shape).

Representative chromatograms for some of the compounds detected in a real river sample are illustrated in Fig. 1A and B for PI and NI mode, respectively.

3.1.2. Optimization of MS/MS parameters

Optimum ionization mode as well as the parent ion and the more abundant fragment ions were chosen for each compound and internal standard trying to get the maximum sensitivity and selectivity. Selection of parent ions and optimum ionization mode for each compound was performed by FIA (Flow Injection Analysis) of 10 μg mL^{-1} individual standard solutions in MeOH/H_2O (25:75, v/v) in full-scan mode at different values of cone voltage (CV). Out of the 74 pharmaceuticals investigated, 57 (plus the corresponding 18 internal standards) showed higher response in PI mode and 17 (plus the corresponding 6 internal standards) in NI mode. Compounds analyzed under NI mode included most of the analgesics and non steroidal anti-inflammatory drugs, barbiturates, furosemide, chloramphenicol and some lipid regulators. In all cases, $[M-H]^-$ for NI and $[M+H]^+$ for PI mode were selected. Further identification of the most or the two most abundant fragment ions (depending on whether it was an internal standard or a regular standard, respectively) was selected in daughter scan of the previously selected parent ion at several collision energies. Finally, the optimum collision energy (CE) was selected for every daughter selected in MRM mode. Table 1 shows the SRM transitions selected, with the optimum CV, CE values for each analyte and transition.

3.1.3. Criteria followed for quantitative analysis

Two SRM transitions between the precursor ion and two most abundant fragment ions were monitored for each compound. The first one was used for quantification purposes (quantification transition) whereas the second one was to confirm the presence of target compounds in the samples (confirmation transition). In this way, the number of identification points (IP) needed to confirm the detection of target analytes, according to the EU regulations (EU Commission Decision 2002/657/EC), are achieved (4IP, 1 for precursor ion and 1.5 for each transition product). The only exceptions were ibuprofen and ketoprofen, for which only one SRM transition could be recorded, due to their poor fragmentation.

Besides the monitoring of the SRM transitions, other identification criteria were used for quantification:

(a) Difference in LC retention time between every compound and its corresponding internal standard within ±2% in both calibration curve solutions and samples.

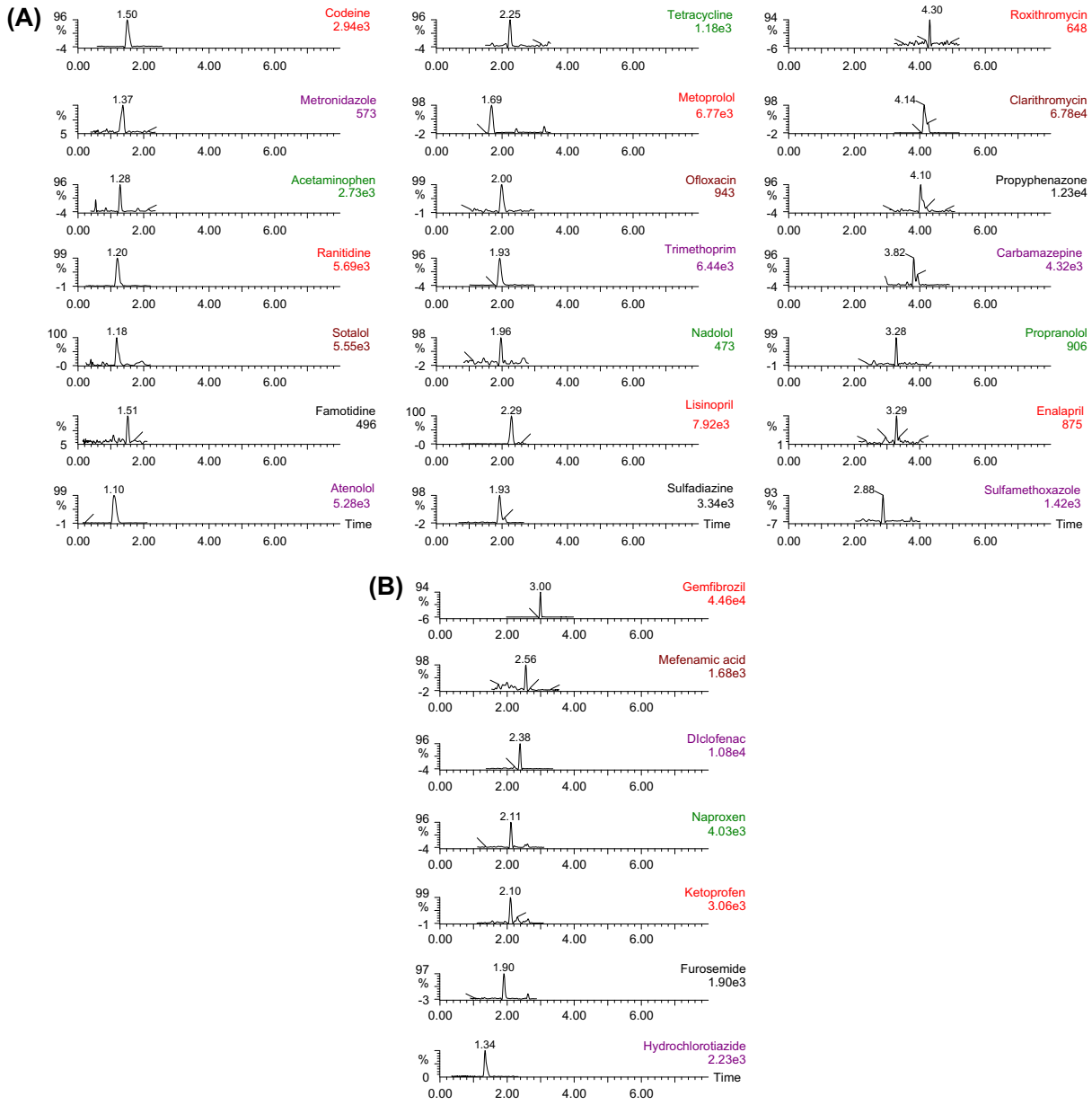

Fig. 1. Chromatograms of some of the compounds detected in T3 for (A) positive and (B) negative mode.

(b) The relative abundances of the two selected analyte SRM transitions in the sample must be within ±20% of the two SRMs ratio in the analytical standards.

Time of retention and relative abundances of the SRM transitions monitored for each compound are also given in Table 1.

3.1.4. Dwell time and acquisition windows optimization

Due to the large number of monitored transitions, acquisition windows were evaluated in order to decrease the number of transitions registered at the same time, and consequently, increase the sensitivity. Thus, a sufficient chromatographic separation was required to get efficient acquisition windows. At the same time, these had to be long enough to be trustworthy in case of a change in the retention time. Finally, 2 min long windows were established both for PI and NI.

Afterward, for every window, dwell time was optimized. Dwell time is the time the detector devotes to register every transition in every monitored cycle. This time must be long enough to obtain a sufficient intensity but, at the same time, it should not be too long in order to have short cycles and thus, to have sufficient points per chromatographic peak (from 10 to 15) to get a precise quantification. This is especially critical in UHPLC analysis where peak widths are very small. The criteria were to assure at least 12 points

of acquisition in the narrowest peak. Dwell time values for every compound are shown in Table 1. Total scan time was of 0.5 and 1 s for NI and PI, respectively.

3.2. Method performance

Extraction recoveries for target compounds were determined for all different matrixes by spiking samples (n = 3) at two levels of concentrations, 20 ng L^{-1} and 100 ng L^{-1} for HPLC grade water, GW and SW and 50 ng L^{-1} and 500 ng L^{-1} for both WWE and WWI. Concentrations after the whole procedure, calculated by internal standard approach, were determined for all the spiked samples as well as a triplicate of every corresponding non-spiked sample. The concentrations obtained for non-spiked samples were subtracted from the ones for the spiked samples. Comparing the resulting concentrations with the initial spiking levels, extraction recoveries, for every compound in each type of water, were calculated. Complete numerical data is given in Table S1 (Supplementary material).

SPE recoveries achieved were in the range of 50–150% for the 80%, 76%, 65%, 61% and 57% of target compounds in HPLC grade water, GW, SW, WWE and WWI, respectively. See % SPE recoveries in Table S1A (Supplementary material). Thus, although there was an impoverishment in the SPE efficiency as the matrix got more complex, the SPE recovery values were still acceptable. Some polar compounds, such as salbutamol, famotidine, atenolol, cimetidine, sotalol and ranitidine, as well as some strongly hydrophobic compounds, such as atorvastatin, tamoxifen, phenylbutazone and mevastatin showed poor SPE recoveries. However, the low recovery was not considered an obstacle for their reliable determination, as their sensitivity and reproducibility were good enough (see MDLs in Table S1B (Supplementary material). Nevertheless, although an internal standard approach is used for the calculation of the recoveries, in cases where the retention time for the compound and the corresponding IS do not coincide in spite of having a similar structure, matrix effect can also influence the determination of the extraction recoveries.

Regarding sensitivity, method detection limits (MDLs) and method quantification limits (MQLs) were determined, for environmental and wastewater samples, as the minimum detectable and quantificable amount of analyte with a signal-to-noise of 3 and 10, respectively. Spiked GW, SW, WWE and WWI samples (n = 3) as indicated above were used for their calculation.

As it can be seen in Table S1B (Supplementary material), MDLs achieved ranged from 0.01 to 5 ng L^{-1}, for most of compounds in HPLC grade water, GW, SW and WWE. For WWI, sensitivity fell but was still acceptable with limits between 0.01 and 50 ng L^{-1} for the majority of compounds. MDLs and MQLs were calculated as a signal-to-noise (S/N) ratio of 3 and 10, respectively, from the chromatograms of real-water samples spiked at the lowest validation level tested, using the quantification transition. Instrumental detection and quantification limits (IDL and IQL) were also estimated. Those ones were estimated as S/N = 3 and 10, respectively, from the chromatograms of standard solutions at the lowest concentration level used for the calibration curve.

To ensure a correct quantification, method and instrumental precision were determined as relative standard deviation (RSD), from solutions (n = 5) of 100 ng L^{-1} spiked HPLC grade water samples, processed with the method here described (method repeatability). The resulting extract solutions were injected every other day (instrumental repeatability). RSD achieved ranged from 0.2 and 5 % for method repeatability, and from 0.1 and 10% for instrumental one, for most of the compounds (see Repeatability in Table S1C (Supplementary material)).

Regarding quantitative performance in terms of dynamic range, linear response generally covered three orders of magnitude. Calibration curves were generated using linear regression analysis and over the established concentration range of 0.1/0.5–250 μg L^{-1}, (depending on the compounds), gave good fits (r^2 > 0.99) (see Linearity in Table S1C (Supplementary material).

Influence of matrix effect in the quantitative LC–MS/MS analysis is widely observed and studied phenomenon (Gros et al., 2009; Jelić et al., 2009). The ESI source is highly susceptible to other components present in the matrix, which may result in a signal suppression or enhancement leading to erroneous results. If relevant ion suppression (or signal enhancement) occurs, appropriate quantitative approaches should be applied for its correction and/or minimization in order to get an accurate quantification. In order to

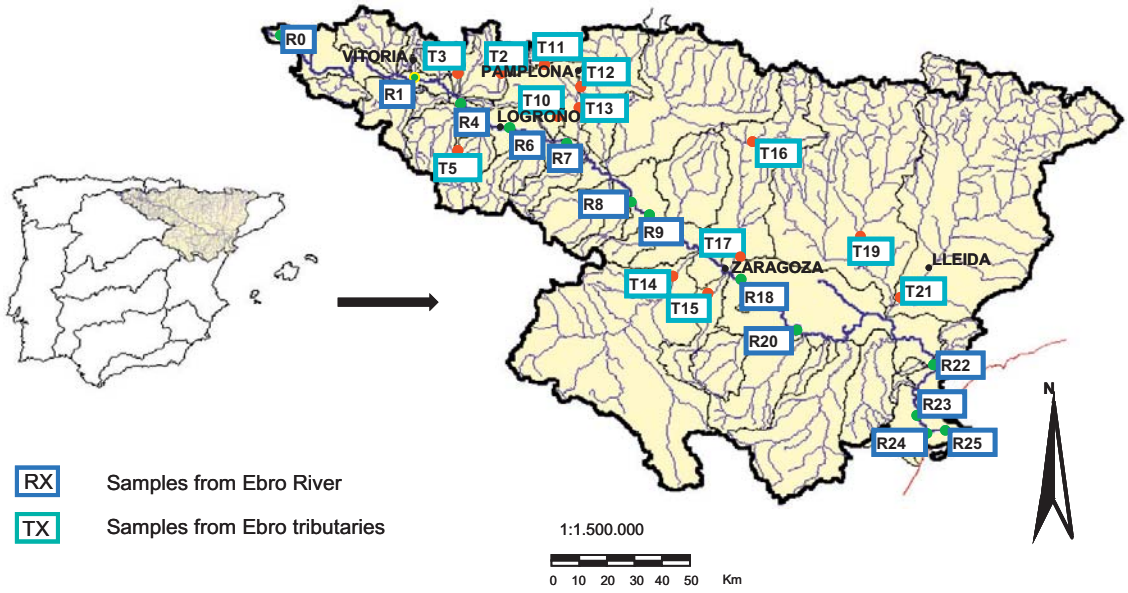

Fig. 2. Sampling location.

Table 2
Range (minimum and maximum) and mean concentration in ng L^{-1} for target pharmaceuticals in surface water in the Ebro River basin (NE Spain).

Therapeutic group	Compound	Min (ng L^{-1})		Max (ng L^{-1})		Mean (ng L^{-1})		Frequency (%)[a]	
		Ebro river	Tributaries	Ebro river	Tributaries	Ebro river	Tributaries	Ebro river	Tributaries
Analgesics and antiinflammatories	Ketoprofen	<MQL[b]	<MQL	106.0	132.5	517.38	53.51	69	62
	Naproxen	<MQL	<MQL	89.11	109.3	29.79	70.15	85	69
	Ibuprofen	4.91	<MQL	43.21	541.9	23.91	96.45	92	100
	Indomethacine	<MQL	<MQL	13.56	62.54	9.84	62.54	38	31
	Diclofenac	<MQL	<MQL	34.39	153.9	14.91	58.34	77	85
	Mefenamic acid	<MQL	<MQL	3.32	9.96	2.28	3.63	100	92
	Acetaminophen	<MQL	<MQL	384.5	872.5	282.4	499.4	62	77
	Salycilic acid	<MQL	<MQL	325.0	446.7	230.8	294.4	100	85
	Propyphenazone	<MQL	<MQL	4.30	48.63	2.66	18.91	92	77
	Phenylbutazone	n.d[c]	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	Phenazone	<MQL	<MQL	<MQL	25.90	<MQL	11.98	8	77
	Codeine	<MQL	<MQL	<MQL	193.7	<MQL	86.84	46	62
Lipid regulators	Clofibric acid	<MQL	<MQL	<MQL	6.03	<MQL	6.03	31	31
	Bezafibrate	<MQL	<MQL	3.02	25.50	2.60	12.69	46	62
	Fenofibrate	<MQL	<MQL	<MQL	1.23	<MQL	1.11	62	85
	Gemfibrozil	1.22	0.91	21.14	212.3	10.48	46.00	92	100
	Mevastatin	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	38	31
	Pravastatin	1.29	1.18	7.47	39.10	3.69	8.57	100	100
	Atorvastatin	<MQL	<MQL	<MQL	53.72	<MQL	28.90	69	92
Psychiatric drugs	Paroxetine	<MQL	<MQL	<MQL	225.0	<MQL	212.5	8	46
	Fluoxetine	<MQL	<MQL	12.00	23.80	10.01	10.87	69	77
	Diazepam	<MQL	<MQL	<MQL	2.68	<MQL	2.68	8	31
	Lorazepam	<MQL	<MQL	11.63	46.13	11.63	31.31	54	46
	Carbamazepine	<MQL	<MQL	8.97	53.80	6.93	29.12	85	38
Histamine H2 receptor antagonists	Loratadine	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	38	23
	Famotidine	<MQL	n.d.	<MQL	n.d.	<MQL	n.d.	8	0
	Ranitidine	<MQL	<MQL	<MQL	109.0	<MQL	86.47	8	38
	Cimetidine	<MQL	<MQL	<MQL	10.70	<MQL	10.70	23	31
Tetracycline antibiotics	Tetracycline	<MQL	<MQL	228.0	86.90	72.74	69.65	46	31
	Doxycycline	<MQL	<MQL	47.70	20.20	20.70	14.06	54	69
	Oxytetracycline	<MQL	<MQL	37.10	26.50	18.58	14.44	92	92
	Chlorotetracycline	5.51	<MQL	59.30	13.70	18.13	9.57	38	38
Macrolide antibiotics	Erithromycin	<MQL	<MQL	15.70	51.60	12.91	26.16	92	85
	Azithromycin	<MQL	<MQL	19.73	36.70	19.73	32.81	23	38
	Tilmicosin	<MQL	<MQL	109.0	227.0	45.78	87.75	85	69
	Roxythromycin	<MQL	<MQL	<MQL	0.32	<MQL	0.32	31	54
	Clarithromycin	<MQL	1.26	11.64	36.93	5.17	8.76	100	100
	Josamycin	<MQL	<MQL	<MQL	0.52	<MQL	0.52	77	69
	Tylosin	<MQL	<MQL	0.72	0.77	0.72	0.77	54	31
	Spiramycin	<MQL	<MQL	242.0	488.0	63.10	129.7	92	85
Sulfonamide antibiotics	Sulfamethoxazol	<MQL	<MQL	<MQL	55.01	<MQL	29.26	15	54
	Sulfadiazine	<MQL	<MQL	23.48	18.43	23.48	16.45	46	31
	Sulfamethazine	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	31	31
Fluoroquinolones	Danofloxacin	<MQL	<MQL	207.0	154.0	72.46	61.16	92	85
	Enoxacin	7.18	<MQL	140.0	104.0	27.31	35.96	92	85
	Ofloxacin	5.24	4.84	105.0	99.60	19.70	37.17	100	100
	Ciprofloxacin	<MQL	<MQL	115.0	93.30	32.57	38.96	100	100
	Enrofloxacin	13.10	14.90	178.0	110.0	38.90	39.55	100	100
	Norfloxacin	<MQL	<MQL	84.60	89.80	22.09	25.52	92	100
	Flumequine	<MQL	<MQL	<MQL	30.29	<MQL	18.87	69	62
Other antibiotics	Trimethoprim	<MQL	<MQL	3.67	29.90	3.67	17.40	38	54
	Nifuroxazide	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	8
	Chloroamphenicol	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	23	38
	Metronidazole	n.d.	<MQL	n.d.	30.20	n.d.	23.80	0	23
Beta blockers	Atenolol	<MQL	<MQL	1238	11020	1031	2789	31	69
	Betaxolol	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	31
	Carazolol	n.d.	<MQL	n.d.	<MQL	n.d.	<MQL	0	8
	Pindolol	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	15
	Nadolol	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	15
	Timolol	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	15	15
	Sotalol	<MQL	<MQL	5.70	423.4	5.70	215.8	38	62
	Metoprolol	<MQL	<MQL	4.27	33.88	4.27	11.51	23	46
	Propranolol	<MQL	<MQL	39.17	14.17	39.17	9.64	15	23
Beta agonists	Salbutamol	n.d.	<MQL	n.d.	<MQL	n.d.	<MQL	0	15
	Clenbuterol	<MQL	<MQL	<MQL	3.42	<MQL	3.42	15	31
Barbiturates	Butalbital	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	15
	Pentobarbital	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	15
	Phenobarbital	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	8

Table 2 (*continued*)

Therapeutic group	Compound	Min (ng L^{-1})		Max (ng L^{-1})		Mean (ng L^{-1})		Frequency (%)[a]	
		Ebro river	Tributaries	Ebro river	Tributaries	Ebro river	Tributaries	Ebro river	Tributaries
Antihypertensives	Enalapril	<MQL	<MQL	0.99	6.84	0.70	4.18	77	69
	Hydrochlorothiazide	<MQL	<MQL	<MQL	571.3	<MQL	203.3	62	62
	Lisinopril	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	46
Diuretic	Furosemide	1.90	1.66	14.60	139.0	8.62	39.60	77	100
Antidiabetics	Glibenclamide	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL	8	31
To treat cancer	Tamoxifen	<MQL	<MQL	<MQL	19.57	<MQL	11.41	46	46

[a] frequency of detection – level above MDL (method detection limit).
[b] MQL – method quantification limit.
[c] n.d. not detected.

evaluate the degree of ion suppression or enhancement in each target compound, matrix effects in all types of validated samples (GW, SW, WWE and WWI) were evaluated by comparing the peak areas from the analysis of spiked real samples (after subtracting the peak areas in the corresponding non-spiked samples), with peak areas from HPLC grade water spiked at the same level. Thus, the percentage of signal suppression was calculated as follows:

$$\%\ \text{Signal suppression}_i = 100 - (((AM_i - AB_i)100)/AH_i) \quad (1)$$

where AM is the peak area of the spiked real sample, AB is the peak area of the corresponding blank, and AH is the peak area of the spiked HPLC water. In our case, it is clearly observed an increase in the suppression effect as the matrix becomes more and more complex. Thus, the 43%, 68%, 77% and 84% of compounds showed suppression in its signal in GW, SW, WWE and WWI, respectively. However, the impact of the matrix is different for every compound. See % signal suppression in Table S1A (Supplementary material). It should be noted that ion suppression/enhancement is different for every sample analyzed even among the same type of samples. Therefore, it is of high significance to use an approach to correct this matrix effect when analyzing real samples. In our study, the approach used was internal standard calibration. Twenty-four isotopically labeled compounds were selected as internal standard for the 74 target analytes, ensuring that all the therapeutic groups and, within them, every family of compounds were represented by at least one suitable internal standard. Out of the 24 IS, 23 corresponded to isotopically labeled compounds of pharmaceuticals included in the present study. The assignments of internal standards can be seen in Table 1.

3.3. Monitoring results

To demonstrate the applicability of the developed method, river water samples from the Ebro basin (NE Spain) were analyzed. Twenty-six sampling sites were selected covering the whole Ebro River Basin (13 at the Ebro River (designated as *RX*) and 13 at the main tributaries (designated as *TX*)). River water was collected downstream of six major WWTPs and other vulnerable sites, according to the proximity to big cities or industrial areas like Miranda de Ebro, Vitoria, Logroño, Pamplona Zaragoza o Lleida. See Table S2 (Supplementary material) and Fig. 2. Samples were collected during the winter 2009–2010 (December 2009 and January 2010). Concentrations found were summarized in Table 2 and Table S3 (Supplementary material).

A wide spectrum of PhACs is detected and the levels were generally in the range of tens to few hundreds of ng L^{-1}. Highest average and maximum concentrations were detected for analgesics and anti-inflammatories such as naproxen, ibuprofen, acetaminophen and salicylic acid with max concentrations of 109, 541, 872 and 447 ng L^{-1} respectively. Other compounds detected at levels higher than 100 ng L^{-1} were psychiatric drug paroxetine, antiulcer ranitidine, fluoroquinolone and macrolide antibiotics, β-blocker atenolol, antihypertensive hydrochlorotiazide and diuretic furosemide. Lipid regulators gemfibrozil, atrovastatin and pravastatin were detected in all samples with average concentrations lower than 50 ng L^{-1}. Compounds occasionally detected (mainly at sampling sites downstream of big urban centers at T3, T12, T15 and T21), or those not found were presented in italics in Table S3 (Supplementary material). This group is composed of analgesics indomethacine and phenylbutazone, the lipid regulators clofibric acid and mevastatin, psychiatric drug diazepam, histamine H2 receptor antagonists, antibiotics nifuroxazide and metronidazole, beta blockers betaxolol, carazolol, pinadolol, nadolol, timolol and propranolol. None of the barbiturates was detected at level higher than MQL.

The results obtained are rather similar to those reported in two previous studies (Gros et al., 2007, 2010), although the average concentrations are generally higher that the previously reported. Main reason is that the present study involves more sampling sites (26 in the present study vs. 9 in the previous ones) including several hot-spots, which may explain higher maximum and mean concentrations detected for the majority of compounds. However, the general pattern of most frequently detected compounds and their geographic distribution are similar to the previously observed.

Regarding to the spatial distribution along the Ebro River, sampling points T3, T12, T15 and T21 were the points where compounds are detected at the highest concentrations. Two of them, T12 and T21 are located downstream of important population centers (cities of Pamplona and Lleida, respectively), while T3 and T15 are important industrial areas. All four sampling points belonged to the Ebro tributaries. In general, the tributary rivers showed higher concentrations for the majority of compounds analyzed, which was attributed to the lower flows and consequently lower dilution factors, as it is shown in Table S2 (Supplementary material). Another observation was that the concentration of pharmaceuticals in the main course of the Ebro River remained stable along the river for most of compounds with no significant increase in concentration from its source to the mouth, even downstream of urban centers such as Logroño (R6) or Zaragoza (R18). The standard deviation for the concentrations found along the Ebro River was lower than 50 ng L^{-1} for most of compounds. A possible explanation to this is increase in dilution along the Ebro river (the average flow during the sampling period was around 100 $m^3\ s^{-1}$ in the upper part of the river and more than 300 $m^3\ s^{-1}$ in the lower course) and natural attenuation.

4. Conclusions

Application of multi-residue methods provide wider knowledge about the occurrence of pharmaceutical residues in the environ-

ment, necessary for further understanding of their removal, partition and ultimate fate. However, simultaneous analysis of compounds from diverse groups with different physico-chemical properties often imposes a compromise in the selection of experimental conditions for their extraction, LC separation and MS detection. The multi-residue analytical method described herein is based on single-step SPE followed by UHPLC–MS/MS detection and allowed simultaneous analysis of 74 multi-class pharmaceuticals in different types of environmental as well as sewage water (river water, ground water, WWTP influent and effluent). The main advantage of the presented method is its high throughput due to the use of fast chromatography (UHPLC). In addition, with the use of a high number of isotopically labeled compounds (24) as internal standard, the method increases also in reliability in comparison to other published methods. The method yielded detection limits in the low ng L^{-1} range for both environmental and wastewaters, what is essential for proper monitoring of the target compounds in those matrices. Application of the method to the analysis of river water samples showed a widespread occurrence of pharmaceuticals in the Ebro river basin, with levels in the range of hundreds of pg L^{-1} and tens of μg L^{-1} depending on the compound and the sampling point, with highest concentrations and frequencies in tributary rivers with low flow rates and low dilution factors.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2011.07.071.

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Publicación científica #2

“Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry”

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Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction–liquid chromatography-electrospray–tandem mass spectrometry

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ABSTRACT

The present work describes the development of a fully automated method, based on on-line solid-phase extraction (SPE)–liquid chromatography-electrospray–tandem mass spectrometry (LC–MS–MS), for the determination of 74 pharmaceuticals in environmental waters (superficial water and groundwater) as well as sewage waters. On-line SPE is performed by passing 2.5 mL of the water sample through a HySphere Resin GP cartridge. For unequivocal identification and confirmation two selected reaction monitoring (SRM) transitions are monitored per compound, thus four identification points are achieved. Quantification is performed by the internal standard approach, indispensable to correct the losses during the solid phase extraction, as well as the matrix effects. The main advantages of the method developed are high sensitivity (limits of detection in the low ng L^{-1} range), selectivity due the use of tandem mass spectrometry and reliability due the use of 51 surrogates and minimum sample manipulation. As a part of the validation procedure, the method developed has been applied to the analysis of various environmental and sewage samples from a Spanish river and a sewage treatment plant.

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1. Introduction

It is estimated that approximately 3000 different substances are used as pharmaceutical ingredients worldwide today. However, only a small subset of these compounds (~150) has been investigated in environmental studies. The worldwide average per capita consumption of pharmaceuticals per year is estimated to be about 15 g, but in industrialized countries the value is much higher and is estimated to be between 50 and 150 g. After administration, most pharmaceuticals are not completely metabolized. The unmetabolized parent drugs and some metabolites are subsequently excreted from the body via urine and faeces [1] reaching the Wastewater Treatment Plants (WWTPs) via wastewater. Reports have shown that many pharmaceuticals do not degrade during municipal conventional wastewater treatment [2–8] being, therefore, discharged to the receiving waters. Recent data indicate that, as much as, 80% of the total load of pharmaceuticals entering a WWTP may be discharged into surface water [9,10]. Disposal of unused or unwanted medications to the toilet or household waste is another route of their entry to the environment.

The concentrations of individual compounds in surface waters are typically in the range of several tens to hundreds of ng L^{-1}, although concentrations at the μg L^{-1} level are also reported for some compounds and specific sites [11]. Generally, these concentrations are lower than typical maximum concentrations (in the tens of μg L^{-1}) reported for some industrial contaminants (e.g. surfactants, plasticizers), but due to their continuous introduction into the environment and bioactive properties, pharmaceuticals may pose a risk to the aquatic organisms and ultimate to humans. One of main concerns is contamination of groundwater through surface water filtration and landfill leakage [1].

Generally, very little is known about the long-term effect and behaviour of pharmaceutical residues in the aquatic environment [12], and in groundwater in particular [13]. In addition, environmental risk assessment is often carried out for individual pharmaceutical compound (active ingredients), while pharmaceutical compounds are typically detected in mixtures with other anthropogenic contaminants [11]. Studies have shown that combinations of pharmaceutical compounds exert a much stronger toxic effect that could be expected from the weak toxic effects related to

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exposure to each compound individually [14,15], i.e., the combination of drugs that share a common mechanism of action exhibits synergistic effects [16].

Therefore, monitoring of wide-range pharmaceuticals in surface and ground waters is as a prerequisite for proper risk assessment. Nowadays, a large number of analytical methodologies, mainly using liquid chromatography–tandem mass spectrometry (LC–MS/MS), are already available for pharmaceutical determination in both environmental and wastewaters [17–19] being antibiotics the most widely studied group [20–22]. Majority of the methods employ rather tedious and time-consuming sample preparation based on off-line solid-phase extraction (SPE). However, the growing number of samples to be analyzed in laboratories carrying out monitoring studies requires employment of high-throughput and fully automated analytical techniques. Because of these reasons, great effort is going into the development of fast, cost-effective and "greener" alternative methods for environmental analysis. Over the past several years, there has been an increase in the use of automated instruments that integrate extraction, purification and detection step (i.e. on-line solid phase extraction systems such as Symbiosis™ and Prospekt-2 systems manufactured by Spark Holland). On line SPE followed by LC–MS/MS that has been used to analyze trace emerging contaminants in water, such as drugs of abuse, pesticides, and hormones [23–27]. With respect to the analysis of pharmaceuticals in aqueous environmental samples several papers were published recently [24,27–29]. For example [28], used on-line SPE in the analysis of six pharmaceutical indicators in water, while [29] reported on the application on line SPE for the analysis of macrolide antibiotics.

In this work, a reliable, fully automated method for the determination of 74 pharmaceuticals in environmental waters (groundwater (GW) and superficial water (SW)) and wastewater (WWTP effluent (WWE) and WWTP influent (WWI) has been developed, validated and applied to real samples. Target compounds, which are listed in Table 1, belong to different medicinal classes and were selected based on their high human consumption, ecotoxicological relevance and ubiquity in the aquatic environment, according to the information found in the scientific literature [30–42].

The objective of this work is to develop an analytical method for simultaneous analysis of a large number of target compounds belonging to different therapeutical classes, that will have clear advantages and improvements over existing methods in terms of (i) minimum sample manipulation; (ii) maximum sensitivity; (ii) reliability, and (iv) selectivity and thus to fulfil the stringent criteria set by the EU regulations (EU Commission Decision 2002/657/EC) [43].

The developed method was successfully applied to the analysis of pharmaceutical residues in WWTP as well as river and drinking water samples.

2. Material and methods

2.1. Chemicals

All pharmaceutical standards were of high purity grade (>90%) and are listed in the Supplementary data 1.

Both individual stock standard and isotopically labelled internal standard solutions were prepared on a weight basis in methanol (MeOH), except fluoroquinolones which were dissolved in a water–methanol (H_2O/MeOH) mixture (1:1) containing 0.2% (v/v) hydrochloric acid, as they are slightly soluble in pure MeOH [44]. After preparation, standards were stored at −20 °C. Special precautions have to be taken into account for tetracycline antibiotics, which have to be stored in the dark in order to avoid their exposure to the light, since it has been demonstrated that they are liable to photodegradation [45]. Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of substances was renewed every three months. On the other hand, compounds with number (see Table 1) 26, 5, 10, 12 and 8, were obtained as solutions in acetonitrile (ACN), while 67 and 65 were dissolved in MeOH, at a concentration of 1 mg mL^{-1}.

A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in MeOH/H_2O (25:75, v/v). Working standard solutions, also prepared in MeOH/H_2O (25:75, v/v) mixture, were renewed before each analytical run. Working solutions were prepared in amber glass vials while standard mixtures were prepared in volumetric flasks wrapped with aluminium foil, in order to prevent the exposure of tetracycline antibiotics to light. A separate mixture of isotopically labelled internal standards, used for internal standard calibration, was prepared in MeOH and further dilutions also in MeOH/H_2O (25:75, v/v) mixture.

HPLC grade MeOH, ACN, water, hydrochloric acid 37% and formic acid 98% were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dehydrate (thereon Na_2EDTA) was 99% from Sigma–Aldrich (Steinham, Germany). Nitrogen for drying 99.995% of purity was from Air Liquide (Madrid, Spain).

2.2. Sample pre-treatment

The method was optimized using groundwater, river water, WWTP influent and effluent. Amber glass bottles pre-rinsed with ultra-pure water were used for sample collection. Water samples were filtered through 1 μm fiberglass filters from Whatman (Fairfield, Connecticut, USA) followed by 0.45 μm nylon membrane filters from Teknokroma (Barcelona, Spain). Na_2EDTA 0.1% (m/v) was added to all samples in order to form complexes with inorganic elements. As it is indicated in [19], this addition improves in a great extent the extraction efficiency of tetracycline, macrolide and fluoroquinolone antibiotics. This could be explained by the fact that these compounds can potentially bind residual metals present in the sample matrix and glassware, resulting in low extraction recoveries [46–50]. The amount of Na_2EDTA added was the same for all types of water analyzed and was considered to be sufficient to enabled formation of complexes with inorganic compounds in all types of matrices, even in waters with high mineral content. Finally, 200 μL of a 0.05 ng μL^{-1} standard mixture containing 37 surrogates for the analysis in positive ion (PI) mode, and 14 surrogates for the analysis in negative ion (NI) mode (see Table 1), were added in every 100 mL of sample for surrogate control and internal standard calibration.

2.3. On-line trace enrichment

Preconcentration of the samples and its chromatographic separation was performed using an automated on-line SPE–LC device Symbiosis™ Pico from Spark Holland (Emmen, The Netherlands). The base of the Symbiosis™ Pico system is a high-end HPLC system with a high performance injector that handles sample volumes from 10 μL up to 10 mL fully automated. This equipment also counts with the Alias™ autosampler that includes positive headspace pressure, extensive wash routines for minimal carry over and 2 injection modes, offline and online SPE. Offline mode was only used in the optimization procedure to assess the recovery by comparing the peak areas obtained in the on-line analyses of spiked waters samples with those obtained from the injection of standard mixtures of the analytes in MeOH/H_2O (25:75, v/v) at equivalent concentrations.

A meticulous experiment design was carried out to optimize SPE (see Table 2A and B). Three different disposable trace enrichment

Table 1
Target compounds organized in their therapeutical groups and their assigned surrogates.

Therapeutic groups	Compounds	Number	CAS Number	Corresponding surrogate
Analgesics/anti-inflammatories (12)	Ketoprofen	1	22071-15-4	*Ketoprofen-13C-d3*
	Naproxen	2	22204-53-1	*Naproxen-d3*
	Ibuprofen	3	15687-27-1	*Ibuprofen-d3*
	Indomethacin	4	53-86-1	*Indomethazine-d4*
	Diclofenac	5	15307-86-5	*Diclofenac-d4*
	Mefenamic acid	6	61-68-7	*Mefenamic acid-d3*
	Acetaminophen	7	103-90-2	*Acetaminophen-d4*
	Salicylic acid	8	69-72-7	*Salicylic acid-α-13C*
	Propyphenazone	9	479-92-5	*Antipyrine-d3*
	Phenylbutazone	10	50-33-9	
	Phenazone	11	60-80-0	
	Codeine	12	76-57-3	*Codeine-d3*
Lipid regulators and cholesterol lowering stain drugs (7)	Clofibric acid	13	882-09-7	*Clofibric acid-d4*
	Bezafibrate	14	41859-67-0	*Bezafibrate-d4*
	Fenofibrate	15	49562-28-9	*Fenofibrate-d6*
	Gemfibrozil	16	25812-30-0	*Gemfibrozil-d6*
	Mevastatin	17	73573-88-3	*Carbamazepine-d10*
	Pravastatin	18	81093-37-0	*Pravastatin-d3*
	Atorvastatin	19	134523-00-5	*Atorvastatin-d5*
Psychiatric drugs (5)	Paroxetine	20	61869-08-7	*Paroxetine-d4*
	Fluoxetine	21	54910-89-3	*Fluoxetine-d5*
	Diazepam	22	439-14-5	*Diazepam-d5*
	Lorazepam	23	846-49-1	
	Carbamazepine	24	298-46-4	*Carbamazepine-d10*
Histamine H2 receptor antagonists (4)	Loratadine	25	79794-75-5	*Loratadine-d4*
	Famotidine	26	76824-35-6	*Famotidine-13C3*
	Ranitidine	27	66357-35-5	*Ranitidine-d6*
	Cimetidine	28	51481-61-9	*Cimetidine-d3*
Tetracycline antibiotics (4)	Tetracycline	29	60-54-8	*Demeclocycline*
	Doxycycline	30	564-25-0	
	Oxytetracycline	31	79-57-2	
	Chlortetracycline	32	57-62-5	
Macrolide antibiotics (4)	Erythromycin	33	114-07-8	*Erythromycin-13C-d3*
	Azithromycin	34	83905-01-5	*Azithromycin-d3*
	Tilmicosin	35	10850-54-0	
	Roxithromycin	36	80214-83-1	*Clarithromycin-N-methyl-d3*
	Clarithromycin	37	81103-11-9	
	Josamycin	38	16846-24-5	
	Tylosin A	39	1401-69-0	
	Spiramycin	40	8025-81-8	*Spiramycin-d3*
Sulfonamide antibiotics (3)	Sulfamethoxazole	41	723-46-6	*Sulfamethoxazole-d4*
	Sulfadiazine	42	68-35-9	*Sulfadiazine-d4*
	Sulfamethazine	43	57-68-1	*Sulfamethazine-d4*
Fluoroquinolones antibiotics (7)	Danofloxacin	44	112398-08-0	*Ofloxacin-d8*
	Enoxacin	45	74011-58-8	
	Ofloxacin	46	82419-36-1	
	Ciprofloxacin	47	85721-33-1	*Ciprofloxacin-d8*
	Enrofloxacin	48	93106-60-6	*Enrofloxacin-d5*
	Norfloxacin	49	70458-96-7	*Norfloxacin-d5*
	Flumequine	50	42835-25-6	*Flumequine-13C3*
Other antibiotics (4)	Trimethoprim	51	738-70-5	*Carbamazepine-d10*
	Nifuroxazide	52	965-52-6	
	Chloramphenicol	53	56-75-7	*Ibuprofen-d3*
	Metronidazole	54	443-48-1	*Metronidazole-hydroxy-d2*
β-Blockers (9)	Atenolol	55	29122-68-7	*Atenolol-d7*
	Betaxolol	56	63659-18-7	
	Carazolol	57	57775-29-8	
	Pindolol	58	13523-86-9	
	Nadolol	59	42200-33-9	
	Timolol	60	26839-75-8	*Timolol-d5*
	Sotalol	61	3930-20-9	*Sotalol-d6*
	Metoprolol	62	37350-58-6	*Metoprolol-d7*
	Propranolol	63	525-66-6	*Propranolol-d7*
β-Agonists (2)	Salbutamol	64	18559-94-9	*Albuterol-d3*
	Clenbuterol	65	37148-27-9	*Clenbuterol-d9*
Barbiturates (3)	Butalbital	66	77-26-9	*Phenobarbital-d5*
	Pentobarbital	67	76-74-4	
	Phenobarbital	68	50-06-6	
Antihypertensives (3)	Enalapril	69	75847-73-3	*Enalapril-d5*
	Hydrochlorothiazide	70	58-93-5	*Hydrochlorothiazide-d2*
	Lisinopril	71	83915-83-7	*Atenolol-d7*
Dirutics (1)	Furosemide	72	54-31-9	*Furosemide-d5*
Antidiabetic (1)	Glibenclamide	73	10238-21-8	*Glyburide-d3*
To trat cancer (1)	Tamoxifen	74	10540-29-1	*Tamoxifen-(N,N-dimethyl-13C2)*

Table 2
Experiments tested during the online SPE optimization procedure.

Type of water	Type of cartridge	Sample extraction volume (mL)	Wash volume after extraction (mL)
(A) Online SPE experiments in HPLC grade water			
HPLC grade water	HySphere Resin GP	1	1
			2
		2.5	1
			2
		5	1
			2
	PRLP-s	1	1
			2
		2.5	1
			2
		5	1
			2
	Oasis HLB	1	1
			2
		2.5	1
			2
		5	1
			2
(B) Online SPE experiments in real aqueous samples			
GW	HySpere Resin GP	1	1
			2
		2.5	1
			2
		5	1
			2
SW		1	1
			2
		2.5	1
			2
		5	1
			2
WWE		1	1
			2
		2.5	1
			2
		5	1
			2
WWI		1	1
			2
		2.5	1
			2
		5	1
			2

cartridges were evaluated for their efficiency in the on-line SPE of the target pharmaceuticals from water: Oasis HLB (macroporous copolymer of divinylbenzene and *N*-vinylpyrrolidone, 30-μm particle size) from Waters Corporation (Milford, Massachusetts, USA), PLRP-s (cross-linked styrenedivinylbenzene polymer, 15–25-μm particle size) from Spark Holland (Emmen, The Netherlands), and HySphere Resin GP (polydivinyl-benzene, 5–15-μm particle size) also from Spark Holland (Emmen, The Netherlands). In order to evaluate which of these three cartridges yielded higher recoveries of target compounds, HPLC grade water was spiked with 100 ng L^{-1} of each target compound. The experiment is summarized in Table 2A. After cartridge conditioning with 2 mL of MeOH and 2 mL of water (flow rate 5 mL min^{-1}), three different sample loading volumes (1, 2.5 and 5 mL) were tested. The flow through the cartridge was in all cases 1 mL min^{-1}. Afterwards and prior to the elution, cartridges were rinsed with HPLC grade water at a flow rate of 5 mL min^{-1} to complete the transfer of the sample and remove interferences such as inorganic salts. Two wash volumes (1 and 2 mL) were tested in order to optimize it. Upon completion of each SPE protocol, the trapped analytes are eluted from the cartridge to the LC column. Two elution modes can be chosen in Symbisis™ Pico device: a "focusing" approach where a pre-selected quantity of solvent or mixture of solvents can be chosen; or a so called "standard" approach, where the full chromatographic gradient passes through the SPE cartridge before being led to the LC column. Due to the elevated number of target compounds and their different chemical properties, the last option is the more appropriate one because of the wide range of polarity given by the mixture of the mobile phases during the gradient. So, the chance of a successful elution is higher. The full eluate is conducted to the LC column where the chromatographic separation and the subsequent detection by the mass spectrometer are carried out. In meanwhile, during the elution, a new cartridge is put in place and pre-concentration of the next sample is simultaneously performed. This kind of configuration allows short cycle times, which in our approach are 30 and 37 min (the duration of the chromatographic run time) for NI and PI mode, respectively.

Once selected the cartridge which yielded the best SPE recoveries, the same extraction and wash volumes trials were carried out on real matrices (GW, SW, WWE and WWI) previously spiked with a standard mixture of target analytes at environmentally realistic concentrations: 20 and 100 ng L^{-1} for GW and SW, respectively, and 50 and 500 ng L^{-1} for WWE and WWI, respectively (see Table 2B). SPE recoveries as well as the method detection limits (MDLs) achieved in each case where the parameters observed to choose the more suitable extraction and wash volumes.

According to the results obtained by preliminary trials, HySphere Resin GP cartridge, 2.5 mL of sample extraction volume and 1 mL of cartridge wash after extraction, were selected for further experiments and analysis of water samples.

2.4. LC-ESI-(QqLIT) MS/MS analysis

For chromatographic separation, an analytical column was used: a reversed-phase Purospher Star RP-18 endcapped column (125 mm × 2.0 mm, particle size 5 μm) from Merck (Dramstadt, Germany) [19]. For MS/MS analyses, Symbiosis™ Pico was connected in series with a 4000QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer equipped with a Turbo Ion Spray source from Applied Biosystems-Sciex (Foster City, California, USA), where mass spectrometry detection is carried out. 4000QTrap is controlled by means of the Analyst 1.4.2 Software from Applied Biosystems-Sciex (Foster City, California, USA) and a companion software appendix for controlling the Symbiosis™ Pico from Spark Holland (Emmen, The Netherlands).

The chromatographic conditions were adapted from an analytical method previously developed and described elsewhere [19]. For PI mode, this involves a flow rate of 0.3 mL min^{-1}, and ACN/0.1% (v/v) formic acid as mobile phases. The proportion of the organic solvent was programmed to increase from 5 to 95% in the first 20 min and then to 100% in the following 2 min; afterward the column was readjusted to the initial conditions. These conditions were held for 10 min to allow re-equilibration of the column before the next injection. The total time of chromatographic analysis (and cartridge elution) is 37 min. In this mode 57 pharmaceuticals are analyzed. For NI mode, this involves a flow rate of 0.2 mL min^{-1}, and ACN:MeOH (1:1, v/v)/H_2O as mobile phases. The proportion of the organic solvent was programmed to increase from 20 to 80% in the first 15 min and then to 90% in the following 2 min; afterward the column was readjusted to the initial conditions by programming the amount of organic solvent to 20% in 3 min. These conditions were held for 10 min to allow re-equilibration of the column before the next injection. The total time of chromatographic analysis (and the cartridge elution) is 30 min. In this mode 17 compounds are analyzed. In both modes, the injection volume was 20 μL.

For quantitative analysis, the ESI-MS/MS method was modified and adapted from [19]. For most of compounds two SRM transitions between the precursor ion and two most abundant fragment ions were monitored (full list of SRMs and instrumental conditions are given in Supplementary data 2). Only one transition was monitored for the isotopically labelled standards since they are added in a concentration elevated enough (100 ng L^{-1}) to be reliably quantified in its more intense transition. In order to obtain additional confirmation, especially for compounds showing poor fragmentation, an Information Dependent Acquisition (IDA) experiment was performed, with SRM as the survey scan and an Enhanced Product Ion Scan (EPI), at three different collision energies, as dependent scan. The obtain spectra were compared with library data based on EPI spectra at the three collision energies used. This allows broad accomplishment of the requirements set by the EU regulations (EU Commission Decision 2002/657/EC) [43] related to identification and confirmation of pharmaceuticals in LC–tandem MS analysis.

Improvements of the existing MS/MS method included: (i) a total of 51 isotopically labelled compounds (37 in PI and 14 in NI mode) were added before the SPE, (ii) an additional compound, the antibiotic flumequine, was included; (iii) a second transition has been tuned for the hydrochlorothiazide, lisinopril, acetaminophen, pravastatin and norfloxacin. For all these ones, the selection of parent ions and optimum ionization mode were performed by infusing 100 μg L^{-1} individual standard solutions in full-scan mode at different values of declustering potential (DP). In all cases, $[M-H]^-$ for NI and $[M+H]^+$ for PI mode were selected. Subsequent identification of the two most abundant fragment ions (one for surrogate standards) and selection of the optimum collision energies (CEs) and collision cell exit potentials (CXP) for each one was carried out in the product ion scan mode, also infusing standard solutions of each individual substance.

In order to obtain enough points per peak to fulfil the European Directive and, at the same time, to get the highest sensitivity possible, the dwell time values were adjusted to 12 in PI (providing a total scan time of 2.15 s) and 31 ms for NI (with a total scan time of 2.12 s), with pauses between ranges of 2 (PI) and 5 ms (NI).

3. Results and discussion

3.1. Solid phase extraction

Three parameters were optimized for the performance of the method in environmental waters (groundwater and superficial water) and sewage water (influent and effluent to a waste water treatment plant (WWTP)): the type of cartridge, the sample extraction volume and the wash volume after extraction. SPE recoveries and method detection limits (MDLs) were the criteria used to make the more appropriate choice for every parameter.

Type of cartridge optimization: Table 2A shown the experimental set up. Extraction recovery of each compound was compared among all the experiments realized for every type of cartridge. For hydrophilic compounds, such as salbutamol, famotidine, sotalol, ranitidine, cimetidine, HySphere Resin GP cartridges are clearly the best performing cartridge. As the hydrophobia increases, the differences among the performing of the three cartridges decrease. For the most hydrophobic compounds (betaxolol, paroxetine, propyphenazone), Oasis HLB cartridges are the ones with better performing, nevertheless, differences with the other two cartridges compared are not significant, especially with HySphere Resin GP. In general, in PI as well as NI mode, the best recoveries (near 100%) were obtained for HySphere Resin GP, for a higher number of compounds. In Fig. 3 extraction recoveries of some representative compounds are shown.

Sample volume optimization: In comparison with conventional methods, where hundreds or even thousands of mL of sample were needed [7,19,51–53], in the present method, much smaller sample size (units of mL) was needed since the whole eluate goes into the analytical column. Three extraction volumes have been tested (1 mL, 2.5 mL and 5 mL). In general, volumes that gave best SPE recovery were 1 and 2.5 mL for PI mode, and 2.5 mL for NI mode. In Fig. 4A extraction yield of some representative compounds is shown.

The next step included experiments with real samples in order to check the influence of the matrix on the required sample volume, and consequently on SPE recoveries and MDLs. Less complex matrices, such as GW and SW showed the same tendency seen in experiments with HPLC grade water (as the hydrophobicity of compounds increases the required volume decreased). For the most hydrophobic compounds, 1 mL was the one that gave the best results. For samples with a complex matrix (WWE and WWI), preference of smaller volumes (1 and 2.5 mL) was even more pronounced. That can be due to signal suppression in the ESI because of the matrix (see Section 2). The bigger volume of sample is extracted, the higher amount of matrix is trapped in the cartridge that subsequently gets to the ESI source. In Fig. 4B and C, SPE recoveries comparing extraction volumes were represented for GW and WWE waters, respectively. In general, 1 and 2.5 mL were the volumes that provided the best recoveries in environmental samples (GW and SW) as well as in sewage waters (WWE and WWI) with no big differences between them, so finally, 2.5 mL was selected as the

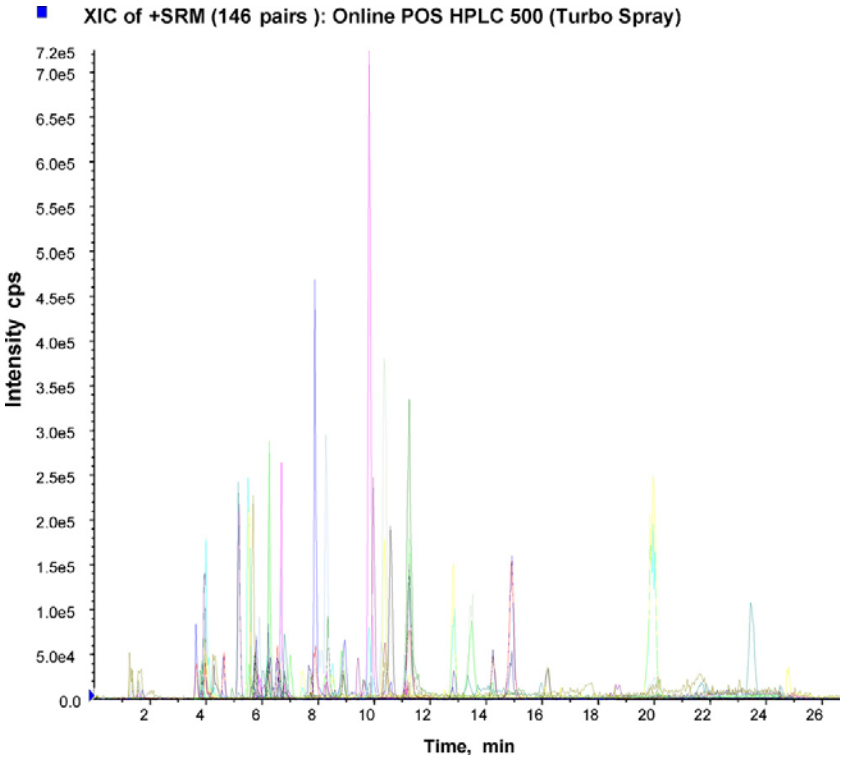

Fig. 1. Chromatogram in positive ESI mode of a HPLC grade water sample spiked with a mixture of standards at 500 ng L^{-1} after being underwent to the online-SPE extraction through GP, 2.5 mL of samples 1 mL of wash.

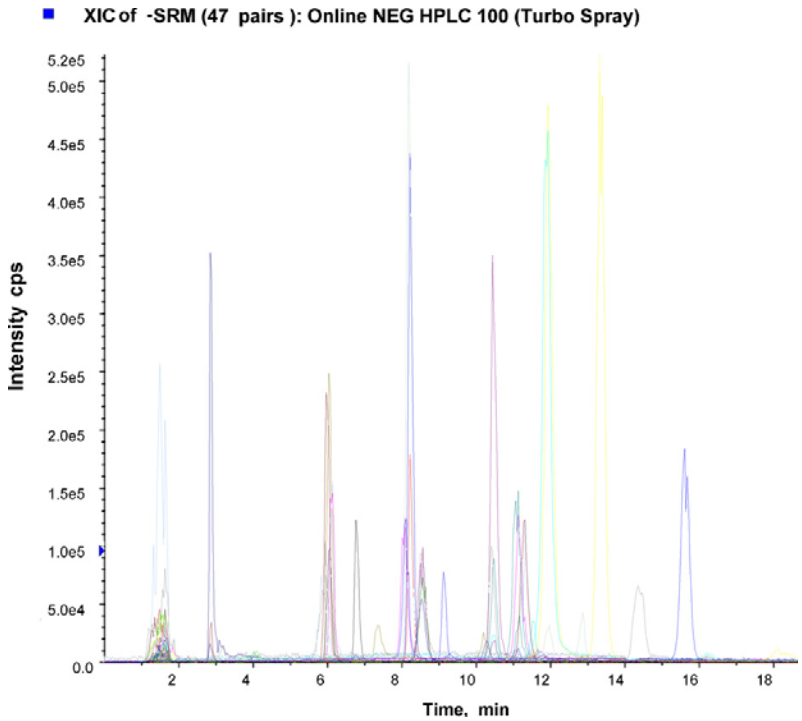

Fig. 2. Chromatogram in negative ESI mode of a HPLC grade water sample spiked with a mixture of standards at 100 ng L^{-1} after being underwent to the online-SPE extraction through GP, 2.5 mL of samples 1 mL of wash.

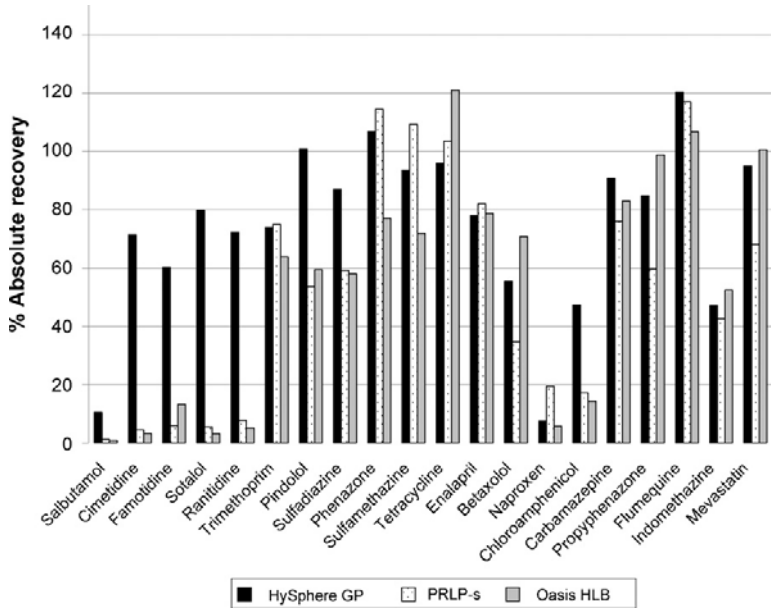

Fig. 3. Cartridge election (HPLC grade water, extraction volume 2.5 mL, wash volume 1 mL).

sample extraction volume, in PI mode as well as in NI one, for all type of samples, since it yielded better MDLs.

Wash cartridge step optimization: Two cartridge wash volumes of water were tested (1 mL and 2 mL). In spiked HPLC grade water samples (experiment in Table 2A), polar compounds gave better SPE recoveries with 1 mL (Fig. 5A). This is consistent with the fact that the solvent used for washing is water, and part of the polar compounds will run with it. For them the less washing volume used the best. For the rest of compounds this parameter is not so influential. In real samples (Table 2B), the same tendency was observed (see Fig. 5B and C). In general, washing with 1 mL of water resulted in best recovery for a higher number of compounds and was chosen for further analyses.

3.2. ESI-(QqLIT) MS/MS detection

Optimization of MS/MS parameters: In the present method, a total of 51 isotopically labelled surrogates (37 in PI and 17 in NI mode) were included which controlled all the steps the samples underwent, in comparison with only 10 internal standards added to the sample after the SPE, just before the LC–MS/MS analysis in [19] where only the matrix effect can be corrected. For a small number of compounds, the corresponding isotopically labelled compound was not commercially available or their price was extraordinarily elevated. An additional compound, the antibiotic forbidden for selling, flumequine is now included in the method. A second transition has been tuned for the hydrochlorothiazide, lisinopril, acetaminophen, pravastatin and norfloxacin improving the reliability of the method compared with [19] where only one transition was registered for those compounds.

Thus, the resulting method includes 125 substances (74 compounds and 51 surrogates), 94 of them (57 compounds and 37 surrogates) monitored in the PI mode and 31 (17 pharmaceuticals and 14 surrogates) in NI mode (Figs. 1 and 2). Transitions between the precursor ion and the two most abundant product ions for each target analyte were recorded for all compounds with the only exception of ibuprofen, phenobarbital, flumequine, ofloxacin, carbamazepine and fenofibrate, for which only one product ion could be obtained. In total, 146 transitions in positive ionization mode (corresponding to 57 compounds and 37 surrogates) and 47 transitions in negative ionization mode (17 compounds and 14 surrogates) were recorded in one single retention time window (Figs. 1 and 2). It should be remarked the fact of that elevated number of transitions were recorded in one single retention time window, without losing sensitivity, due to the setting of appropriate values for the dwell time and pause between mass ranges. Adjusting the dwell time to an appropriate value is a key parameter to monitor large number of transitions in the same time segment and still obtain enough points per chromatographic peak (>15), which is very important for a precise quantification. Dwell time in NI (31 ms) was higher than in PI (12 ms) because the number of transitions was lower, so the detector can devote more time in monitoring every transition in each cycle. Nevertheless, the ionization in PI is better than in NI mode, so the sensitivity for both modes is similar.

3.3. Method performance

Extraction recoveries for target compounds were determined for all different matrices by spiking samples ($n = 3$) at two levels of concentrations 20 ng L^{-1} and 100 ng L^{-1} for HPLC grade water, GW and SW and 50 ng L^{-1} and 500 ng L^{-1} for both WWI and WWE. Those levels were chosen as typical low and high concentrations for most of compounds in those types of waters. For each type of water samples, recoveries were determined by comparing the concentrations obtained after the whole procedure, calculated by internal standard calibration, with the initial spiking levels. As real samples (ground, surface and wastewaters) already contained target compounds, non-spiked samples were analysed in order to determine their concentrations, which were afterwards subtracted to the spiked samples. Due to huge quantity of data, and in order to be easily observed, validation parameters are presented in figures (see Figs. 6 and 7). Complete numerical data is given in Supplementary data 3. Two types of SPE recoveries are provided. Absolute recoveries, determined by comparing the peak areas obtained for spiked water samples in the on-line Symbiosis™ Pico mode of work-

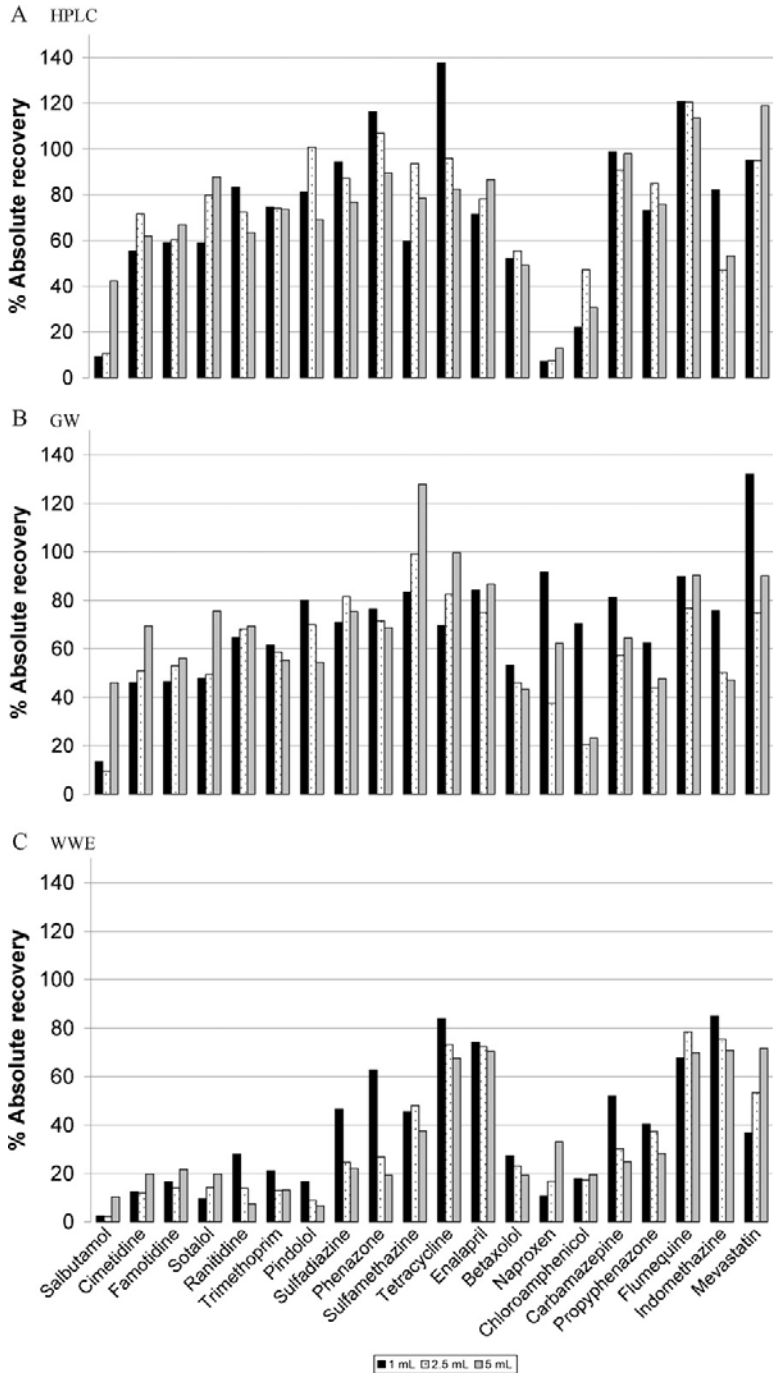

Fig. 4. Extraction volume election (GP cartridge, wash volume 1 mL).

ing, with those achieved from the injection of standards mixtures of the analytes in MeOH/H_2O (25:75, v/v) through off-line mode at equivalent concentrations. Relative recoveries were calculated afterwards by comparing absolute recoveries for every compound and its respective surrogate.

Absolute recoveries achieved were in the range of 50–150% for the 70%, 73%, 61%, 42% and 36% of target compounds in HPLC grade water, GW, SW, WWE and WWI, respectively. See Supplementary data 3A. Thus, it was clear that as the matrix was more complex, the extraction performance and/or the mass spectrometry detection got worse. For polar compounds, as salbutamol, atenolol, cimetidine, famotidine low absolute SPE recovery is obtained (10.1%, 46.0%, 14.4% and 29.2%, absolute recovery in HPLC grade water, respectively). The poor affinity for the cartridge and/or the removal

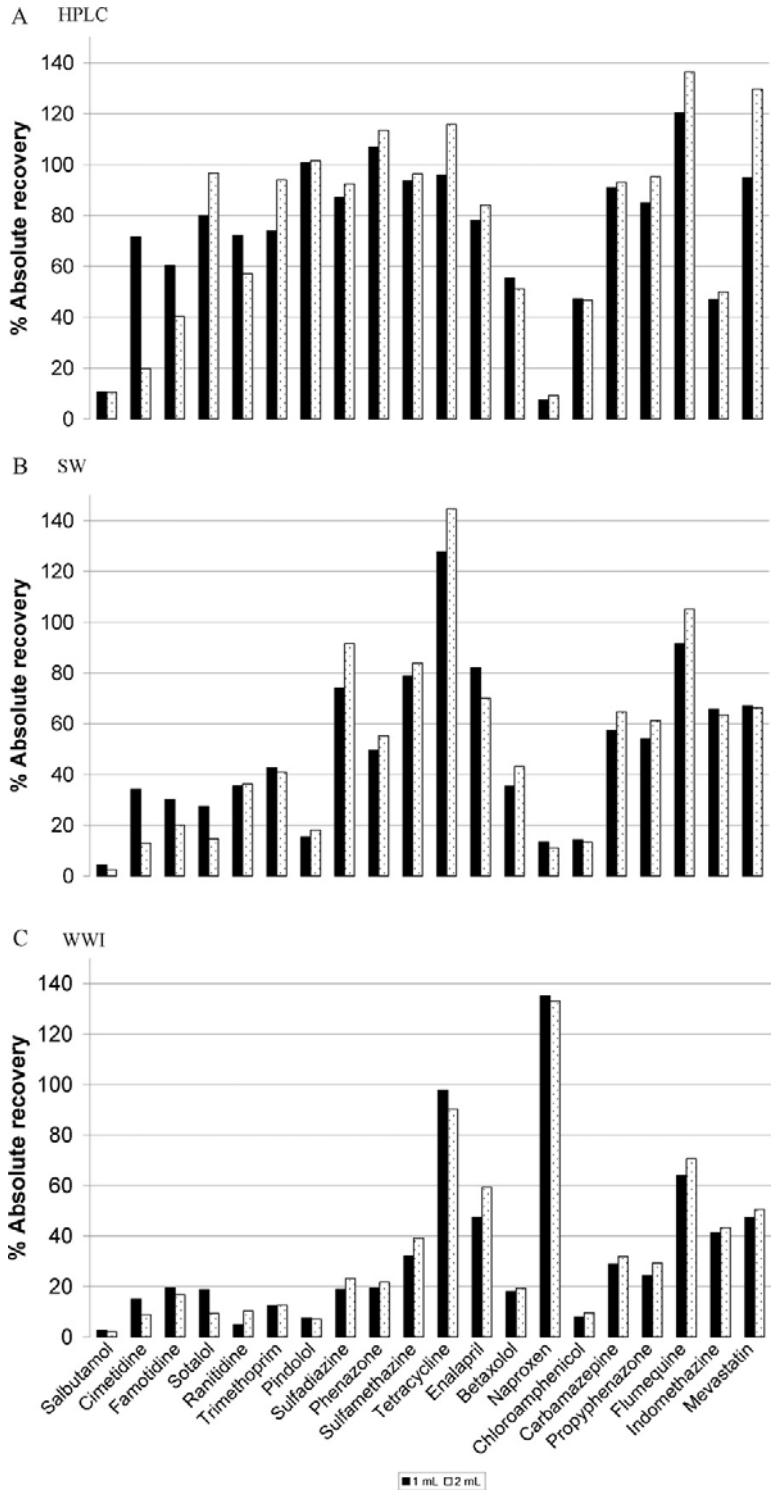

Fig. 5. Cartridge wash volume election (GP cartridge, extraction volume 2.5 mL).

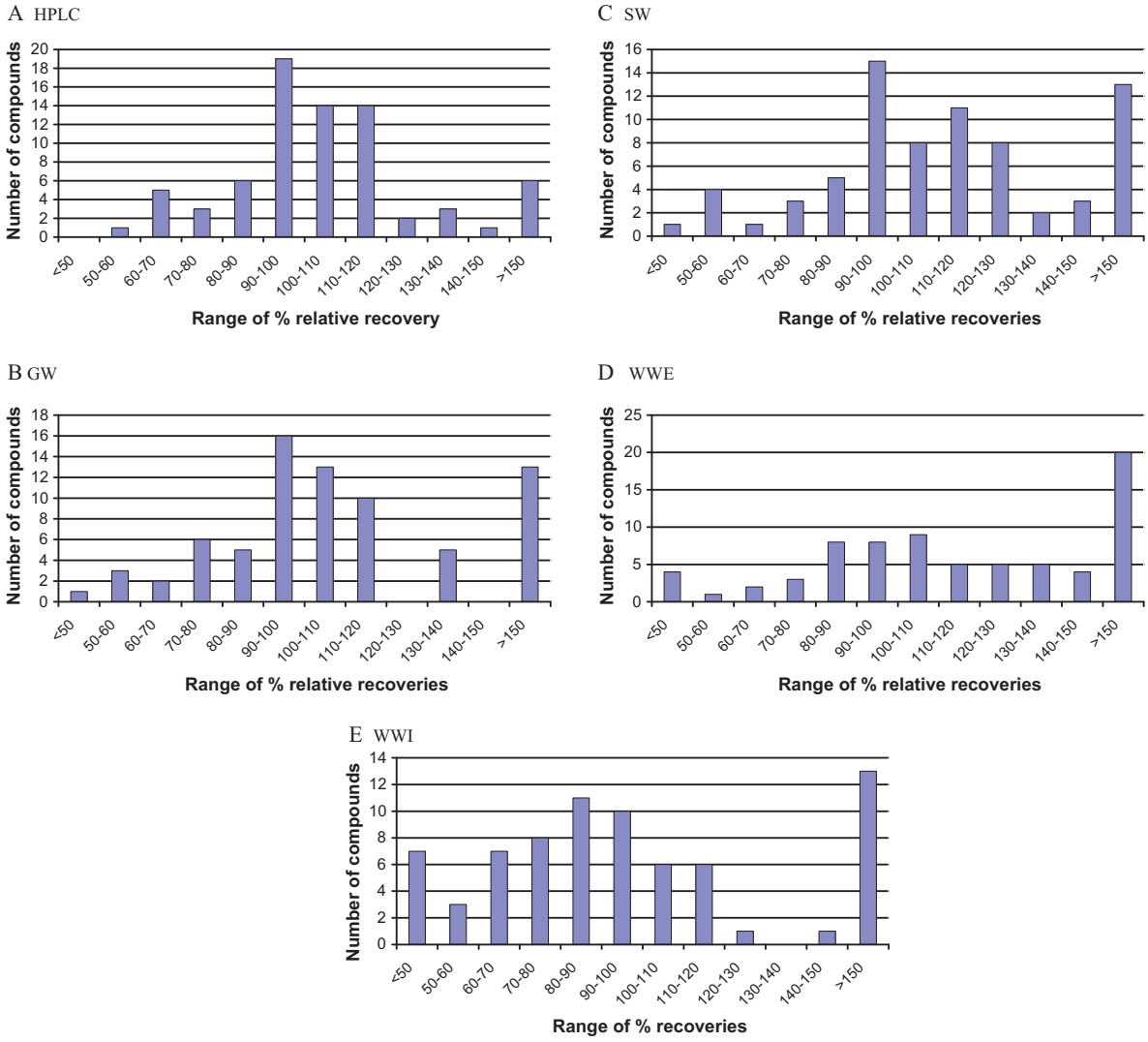

Fig. 6. Relative SPE recoveries organized in ranges for HPLC grade water, GW, SW, WWE and WWI.

from it during the cartridge wash step was the reason of those low absolute recoveries, as no matrix was involved. For chloramphenicol, the absolute SPE recoveries were 87.1%, 82.5%, 78.9%, 28.9% and 16.3% for HPLC grade water, GW, SW, WWE and WWI, respectively. In this case, a clear influence of matrix on the extraction and MS/MS detection occurred. Anyhow, when the SPE recoveries were corrected by the ones for the corresponding surrogates, the percentages of compounds with relative SPE recovery around 100% increased significantly. In this manner, the 92%, 81%, 81%, 68% and 72% of compounds showed a relative SPE recovery between 50 and 150%. Thereby, 111.2%, 117.4%, 97.6% and 122.5% were the relative SPE recoveries, for the same polar compounds named before, respectively. And, 98.0%, 99.2%, 76.1%, 74.4 and 89.8% were the relative SPE recoveries for the chloramphenicol in HPLC grade water, GW, SW, WWE and WWI, respectively. Consequently, poor percentages of absolutely recovery were not considered an obstacle for their reliable determination in water, as their sensitivity was fairly good for being corrected by the corresponding surrogate. The overall method precision, calculated as the relative standard deviation (RSD) was satisfactory, with RSD values ranging from 1 to 30% for most of the compounds in all matrices.

Regarding sensitivity, Method Detection Limits (MDLs) and Method Quantification Limits (MQLs) were determined, for environmental and wastewater samples, as the minimum detectable amount of analyte with a signal-to-noise of 3 and 10, respectively. Spiked GW, SW, WWE and WWI samples ($n = 3$) at the two level of concentrations indicated before were used for their calculation. As it can be seen in the Fig. 7 and Supplementary data 3B, MDLs achieved ranged from 0.01 to 5 ng L^{-1} for most of compounds in HPLC grade water, GW and SW, and from 0.01 to 20 ng L^{-1} for the majority of them in wastewaters.

To ensure correct quantification, precision of the chromatographic method, determined as relative standard deviation (RSD), was determined from repeated injections ($n = 5$) of a 100 ng L^{-1} spiked HPLC grade water sample during the same day (repeatability) and on different days (reproducibility). RSD achieved were lower than 20 and 30% for most of compounds for intra- and inter-day analysis, respectively.

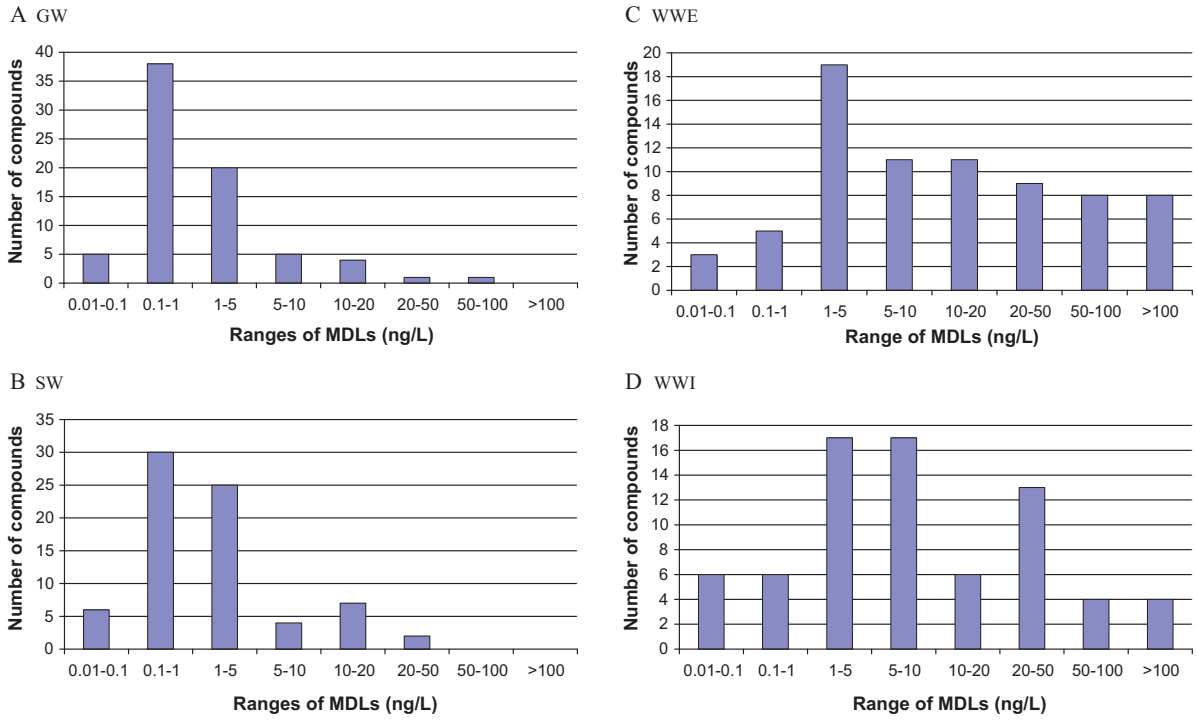

Fig. 7. MDLs organized in ranges for GW, SW, WWE and WWI.

Regarding quantitative performance in terms of dynamic range, linear response covered, giving good fits ($r^2 > 0.99$), four and even five orders of magnitude for the majority of compounds. Calibration curves were generated in HPLC grade water and linear regression analysis was used over the concentration range of 0.01–10,000 ng L^{-1}. Only, glibenclamide, phenyl-butazone, propyphenazone and diclofenac showed a narrower linear response from their MQLs to 500 ng L^{-1}. Thanks to that wide range of linearity, no sample dilution is needed for highly concentrated samples before performing the analysis in order to get a concentration inside the lineal range. For quantification purposes, the internal standard calibration approach was used, performing thirteen-point calibration standards daily, and the possible fluctuation in signal intensity was checked by injecting a standard solution at two concentration levels after each 8–10 injections.

Influence of matrix effect in the quantitative LC–MS/MS analysis is a widely observed and studied phenomena [19,25,54]. The ESI source is highly susceptible to other components present in the matrix, which may result in a signal suppression or enhancement leading to erroneous results. Natural organic matter, salts, ion-pairing agents, non-target contaminants have shown to be responsible for ion suppression. The more complex is the matrix the stronger matrix effect will be present. Therefore, any analytical method where MS is used as detection technique should include a matrix effect study, especially if it deals with complex samples, as in the present case, wastewaters. If relevant ion suppression (or signal enhancement) occurs, appropriate quantitative approaches should be applied for its correction and/or minimization in order to get an accurate quantification. The most common approaches consist of the use of suitable calibration, such as external calibration using matrix-matched samples, standard addition or internal standard calibration with structurally similar unlabelled pharmaceuticals or isotopically labelled standards, as well as dilution of sample extracts [55–58]. In order to evaluate the degree of ion suppression or enhancement in each target compound, matrix effects in all types of validated samples (GW, SW, WWE and WWI) were evaluated by comparing the peak areas from the analysis of spiked real samples (after subtracting the peak areas corresponding to the native analytes present in the sample), with peak areas from spiked HPLC grade water. In the absence of matrix effects, analyte peak areas should be similar in both HPLC grade water and real samples. Nevertheless, when matrix effects occurs the signal intensity for the analytes decreases (ion suppression) or increases (enhancement). Matrix effect was quantified comparing the areas of compounds in spiked matrix samples with the areas obtained in spiked solvent. The effect was expressed by percentage of signal suppression (positive value) or enhancement (negative values). It is clearly observed an increase in the effect as the matrix becomes more and more complex. However, the impact of the matrix is different for every compound. Two extreme examples were bezafibrate, for which rather low effect (−1.07%, 5.72%, 39.06% and 34.51% of matrix effect in GW, SW, WWE and WWI, respectively) is observed, in comparison to phenobarbital for which a much stronger effect was evidenced with 10.90%, 24.54%, 57.48% and 84.85% ion suppression for the same samples. It should be noticed that ion suppression/enhancement is different for every sample analysed even among the same type samples. Therefore, it is of high significance to use any of the aforementioned approaches to correct ion suppression in order to avoid inaccurate quantification and underestimate levels of compounds when analyzing real samples. In our study, the approach used was internal standard calibration. In general, a corresponding isotopically labelled internal standard was selected for each compound (51 surrogates for 74 target analytes). Thus, all the therapeutic groups and within them every family of compounds count with at least an internal standard. The assignation of an appropriate internal standard for

Table 3
Average concentrations and relative standard deviation (expressed in brackets) for target pharmaceuticals in drinking water, superficial water (2 points) and effluent wastewater in the Llobregat River basin (NE Spain).

Therapeutic groups	Compounds	Concentration (ng L^{-1})[a]			
		Point #1 – Llobregat River upstream to the spill point	**Point #2** – Llobregat River downstream to the spill point	**Point #3** – drinking water	**Point #4** – WWE tertiary treatment
Analgesics and antiinflammatories	**Ketoprofen**	*n.d.*	*3.18 (1.56)*	*n.d.*	*57.73 (0.55)*
	Naproxen	**81.05 (0.27)**	**67.38 (0.27)**	**n.d.**	**72.17 (0.33)**
	Ibuprofen	**186.68 (0.33)**	**134.75 (0.32)**	**3.71 (0.15)**	**43.57 (0.60)**
	Indomethacin	**16.27 (0.18)**	**37.75 (0.29)**	**n.d.**	**93.88 (0.52)**
	Diclofenac	**89.53 (0.25)**	**176.78 (0.31)**	**n.d.**	**421.50 (0.26)**
	Mefenamic acid	*n.d.*	*6.76 (0.30)*	*12.82 (2.24)*	*17.38 (0.63)*
	Acetaminophen	**307.00 (0.59)**	**146.67 (0.91)**	**n.d.**	**77.83 (1.77)**
	Salicylic acid	**208.17 (0.07)**	**333.17 (0.61)**	**201.20 (0.23)**	**674.33 (0.26)**
	Propyphenazone	*3.25 (1.11)*	*11.10 (0.68)*	*n.d.*	*22.55 (0.88)*
	Phenylbutazone	n.d.	n.d.	n.d.	n.d.
	Phenazone	*5.90 (1.57)*	*40.27 (0.58)*	*n.d.*	*56.30 (0.28)*
	Codeine	**45.85 (0.8)**	**109.68 (0.41)**	**n.d.**	**350.12 (0.45)**
Lipid regulators	**Clofibric acid**	8.40 (0.36)	24.25 (1.10)	n.d.	22.43 (0.16)
	Bezafibrate	**15.89 (0.34)**	**67.32 (0.47)**	**0.11 (2.24)**	**217.50 (0.50)**
	Fenofibrate	23.85 (0.36)	82.08 (0.49)	n.d.	293.67 (0.64)
	Gemfibrozil	**1.90 (0.39)**	**2.14 (0.57)**	**n.d.**	**8.58 (0.54)**
	Mevastatin	n.d.	n.d.	n.d.	n.d.
	Pravastatin	n.d.	n.d.	n.d.	n.d.
	Atorvastatin	*2.99 (1.19)*	*2.39 (1.10)*	*27.60 (2.24)*	*2.71 (1.32)*
Psiquiatric drugs	**Paroxetine**	*n.d.*	*n.d.*	*n.d.*	*7.30 (0.91)*
	Fluoxetine	*n.d.*	*<LOQ*	*2.74 (2.24)*	*15.87 (0.25)*
	Diazepam	*n.d.*	*6.52 (0.64)*	*n.d.*	*18.92 (0.23)*
	Lorazepam	**22.58 (0.14)**	**41.27 (0.23)**	**n.d.**	**114.92 (0.26)**
	Carbamazepine	**31.28 (0.29)**	**58.43 (0.30)**	**n.d.**	**156.83 (0.24)**
Histamine H2 receptor antagonists	**Loratadine**	*3.68 (1.68)*	*2.51 (0.80)*	*10.48 (1.14)*	*6.99 (1.08)*
	Famotidine	n.d.	n.d.	n.d.	n.d.
	Ranitidine	**33.87 (0.40)**	**61.23 (0.70)**	**n.d.**	**197.67 (1.20)**
	Cimetidine	*17.33 (2.45)*	*n.d.*	*n.d.*	*32.05 (1.17)*
Tetracycline antibiotics	**Tetracycline**	*n.d.*	*29.00 (0.91)*	*n.d.*	*171.47 (1.06)*
	Doxycycline	n.d.	n.d.	n.d.	n.d.
	Oxytetracycline	*n.d.*	*n.d.*	*n.d.*	*42.12 (0.86)*
	Chlorotetracycline	n.d.	n.d.	n.d.	n.d.
Macrolide antibiotics	**Erythromycin**	**50.38 (0.55)**	**174.73 (0.42)**	**n.d.**	**677.00 (0.28)**
	Azithromycin	**14.73 (0.34)**	**71.67 (0.70)**	**17.00 (0.58)**	**1031.67 (0.53)**
	Tilmicosin	n.d.	n.d.	n.d.	n.d.
	Roxythromycin	*n.d.*	*n.d.*	*n.d.*	*3.90 (0.43)*
	Clarithromycin	**42.60 (0.27)**	**88.83 (0.35)**	**3.67 (0.22)**	**237.83 (0.23)**
	Josamycin	*1.82 (0.60)*	*0.81 (1.56)*	*1.41 (0.77)*	*3.03 (0.58)*
	Tylosin	*n.d.*	*n.d.*	*n.d.*	*7.17 (0.57)*
	Spiramycin	**39.90 (0.44)**	**68.32 (0.44)**	**20.54 (0.92)**	**141.58 (0.33)**
Sulfonamide antibiotics	**Sulfamethoxazol**	**39.70 (0.23)**	**78.38 (0.37)**	**n.d.**	**140.48 (0.46)**
	Sulfadiazine	*n.d.*	*13.40 (1.17)*	*n.d.*	*20.38 (0.56)*
	Sulfamethazine	*1.68 (2.45)*	*112.27 (1.55)*	*4.08 (2.24)*	*373.84 (1.92)*
Fluoroquinolones	**Danofloxacin**	n.d.	n.d.	n.d.	n.d.
	Enoxacin	*4.83 (1.56)*	*4.65 (1.57)*	*16.04 (0.96)*	*8.27 (1.13)*
	Ofloxacin	**23.28 (0.24)**	**75.017 (0.48)**	**15.30 (0.73)**	**276.67 (0.46)**
	Ciprofloxacin	**8.32 (0.79)**	**28.02 (0.90)**	**13.28 (0.68)**	**151.25 (1.43)**
	Enrofloxacin	**5.82 (0.56)**	**40.12 (0.84)**	**18.93 (0.79)**	**255.67 (0.35)**
	Norfloxacin	**15.83 (0.31)**	**15.17 (0.86)**	**32.88 (0.94)**	**63.72 (1.02)**
	Flumequine	n.d.	n.d.	n.d.	n.d.
Other antibiotics	**Trimethoprim**	**16.43 (0.24)**	**33.53 (0.34)**	**0.51 (2.24)**	**65.92 (0.37)**
	Nifuroxazide	n.d.	n.d.	n.d.	n.d.
	Chloroamphenicol	n.d.	n.d.	n.d.	n.d.
	Metronidazole	*n.d.*	*44.88 (0.92)*	*n.d.*	*211.83 (0.74)*
Beta blockers	**Atenolol**	**38.40 (0.43)**	**63.17 (0.48)**	**n.d.**	**117.82 (0.56)**
	Betaxolol	n.d.	n.d.	n.d.	n.d.
	Carazolol	n.d.	n.d.	n.d.	n.d.
	Pindolol	n.d.	n.d.	n.d.	n.d.
	Nadolol	n.d.	n.d.	n.d.	n.d.
	Timolol	n.d.	n.d.	n.d.	n.d.
	Sotalol	**15.28 (0.30)**	**44.32 (0.94)**	**n.d.**	**91.98 (0.30)**
	Metoprolol	**54.47 (0.15)**	**327.40 (1.91)**	**38.48 (0.30)**	**96.8 (0.25)**
	Propranolol	*n.d.*	*14.94 (0.39)*	*n.d.*	*51.60 (0.30)*
Beta agonists	**Salbutamol**	*n.d.*	*4.87 (1.70)*	*n.d.*	*27.05 (0.30)*
	Clenbuterol	n.d.	n.d.	n.d.	n.d.
Barbiturates	**Butalbital**	n.d.	n.d.	n.d.	n.d.
	Pentobarbital	n.d.	n.d.	n.d.	n.d.
	Phenobarbital	n.d.	n.d.	n.d.	n.d.

Table 3 (*Continued*)

Therapeutic groups	Compounds	Concentration ($ng\,L^{-1}$)[a]			
		Point #1 – Llobregat River upstream to the spill point	**Point #2** – Llobregat River downstream to the spill point	**Point #3** – drinking water	**Point #4** – WWE tertiary treatment
Antihypertensives	**Enalapril**	n.d.	n.d.	n.d.	n.d.
	Hydrochlorothiazide	**3.26 (0.16)**	**7.96 (0.32)**	**1.26 (0.05)**	**33.53 (0.28)**
	Lisinopril	n.d.	n.d.	n.d.	n.d.
Diuretic	**Furosemide**	**50.98 (0.50)**	**173.72 (0.63)**	**n.d.**	**1120.33 (0.88)**
Antidiabetics	**Glibenclamide**	*0.46 (1.55)*	*1.72 (0.87)*	*3.51 (1.00)*	*13.13 (0.14)*
To treat cancer	**Tamoxifen**	n.d.	n.d.	n.d.	n.d.

[a] values below the limit of detection and below the limit of quantification were considered 0 to calculate the mean value and the RSD.

substances without a specific one, was based on the similarity of their chemical structures and/or their retention times. In Table 1 and Supplementary data 2, internal standards used for each substance, which in this method work as surrogates, are indicated. In this way, the limitation in the number of internal standards presented in [19] was clearly overcome.

3.4. Monitoring results

To demonstrate the applicability of the developed method, two river waters from the Llobregat River (NE Spain), one WWE and the effluent of a drinking water treatment plant (DWTPE) were analyzed River samples correspond to Llobregat River (NE Spain) in two strategic sites up- (point #1) and downstream (point #2) to the point of discharge of treated waters form one WWTP respectively. Point #2 coincides also with the entrance to the DWTP, which was located a few kilometres downstream to the point #2. Point #3 corresponds to the effluent of DWTP (drinking water) and point #4 the WWE after the tertiary treatment which was recirculated towards the discharge point (Fig. 8). Samples from all four points were collected twice a week during three consecutive weeks (six samples per point) during November 2009. The object of this sampling was monitoring the feasibility in the reuse of WWE after a tertiary treatment. Despite the point #3 did not correspond to a specific type of water validated for this method, it was considered similar to a groundwater because of their poor matrix and the low levels of pharmaceuticals expected.

Average concentration for the six samples per point is summarized in Table 3. Levels detected were in the range of hundreds of $pg\,L^{-1}$ to low tens of $ng\,L^{-1}$ for drinking water, and up to low hundreds of $ng\,L^{-1}$ for surface water. Levels in wastewater effluent samples were from units to hundreds of $ng\,L^{-1}$ depending on the compound or even thousands of $\mu g\,L^{-1}$ in some cases such as the antibiotic azithromycin and the diuretic furosemide. Data from the most frequently detected and at higher concentration compounds is presented in bold. Antibiotics, analgesics and anti-inflammatories were the most ubiquitous compounds. The azithromycin and diclofenac must be remarked among them, respectively. As expected, higher concentration were shown at point #4 (WWE after the tertiary treatment). For the diuretic furosemide, this concentration was especially elevated ($1120\,ng\,L^{-1}$), but after the spill into the river, the concentration decreased in a great extent ($173\,ng\,L^{-1}$). Anyhow, that concentration was still higher regarding to the one in the river upstream in the point #1 ($51.0\,ng\,L^{-1}$). This tendency was observed for most of compounds. So it can be said that, after the discharge of effluent

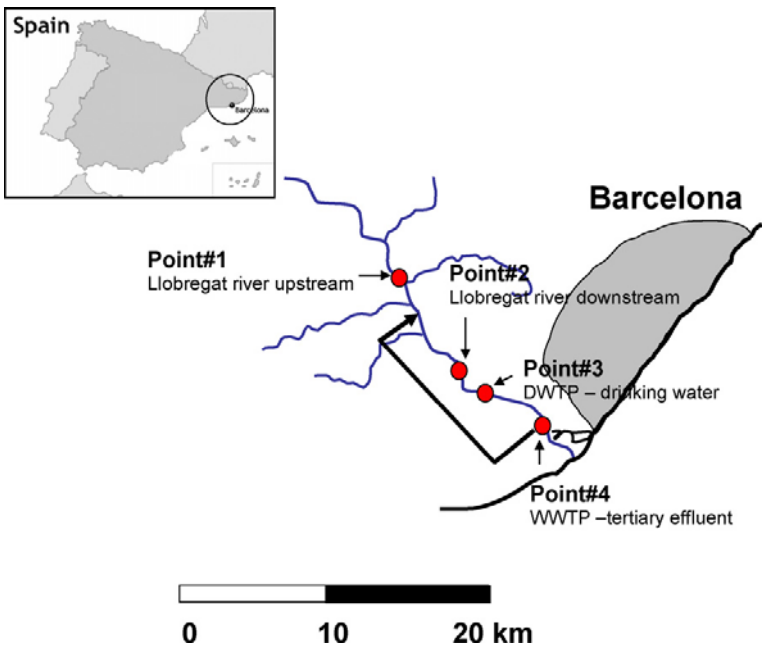

Fig. 8. Sampling location.

the dilution effect is quite effective, but anyway, the perturbation can be observed. However, the decrease in the levels of concentration after the discharge into the river is not the same for all the compounds, even taking into consideration the quantity already present in the river upstream. Thus, in addition to the dilution which is physical phenomenon which should affect all compounds in the same extent, other process like adsorption to sediments or suspended solids, biodegradation or even photodegradation must be taken into account. Anyhow, levels of pharmaceutical at the entrance of the DWTP (point #2) were low and after the treatment at DWTP (point #3) drinking water contained undetectable or very low concentrations for most pharmaceuticals, with the exception of salicylic acid that was detected at 200 ng L^{-1}.

Compounds occasionally detected or detected at low levels even at point #4 were presented in italics. For some of them, quantifications were only possible at point #4. But after the discharge into the river the levels decreased under the limits of quantification or even detection. The macrolides tylosin and roxythromycin, and the cycline oxytetracycline were some examples of that. The presence of compounds, whose quantification at point #2 was still got, could be attributed exclusively to the discharge from the WWTP. In those cases, purification in the DWTP treatment was responsible for reducing their levels down the limit of quantification and/or detection, (e.g. 20–30 ng L^{-1}). 23 compounds were not detected in any sample at any point of sampling.

4. Conclusions

The fully-automated multi-residue analytical method developed, based on on-line SPE–LC–MS/MS allowed the analysis of 74 multiple-class pharmaceuticals in two environmental types of water as well as waste water (influent and effluent to a WWTP). Since the SPE is carried out fully automated, on-line and simultaneously to the chromatographic separation and mass spectrometry detection, a minimum sample manipulation is involved, and therefore a clear decrease in the error introduction is achieved. In fact, filtration is the only sample pre-treatment required. In this way, the method increases in reliability in comparison with conventional off-line methods. To this feature also contributes the fact that most of compounds count with a specific isotopically labelled compound as surrogate (quasi isotopic dilution approach). The method yielded detection limits in the low ng L^{-1} range for both environmental and wastewaters, what is essential for proper monitoring of the target compounds in those type of samples. Moreover, regarding to selectivity, the method fulfil the stringent criteria set by the EU regulations (EU Commission Decision 2002/657/EC) [43]. Other advantages of this method is its high throughput (total analysis time is 30 min in NI mode and 37 min in PI mode) and the wide linear range for most of compounds, which avoids the necessity of diluting the samples for determining compounds present at higher concentrations. It must also be remarked the small size of sample needed, 2.5 mL per ionization mode (total of 5 mL), what relieves the storage problems so usual in analytical laboratories. Application of the method to the analysis of drinking, surface and effluent wastewaters showed a widespread occurrence of pharmaceuticals in such matrices, with general levels, when detected, in the range of units and tens of ng L^{-1} for drinking and river water, respectively, and tens and hundreds of ng L^{-1} in wastewaters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.09.046.

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Publicación científica #3

"Direct analysis of pharmaceuticals, their metabolites and transformation products in environmental waters using on-line TurboFlowTM chromatography-liquid chromatography-tandem mass spectrometry"

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Rebeca López-Serna, Mira Petrović, Damià Barceló

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Direct analysis of pharmaceuticals, their metabolites and transformation products in environmental waters using on-line TurboFlow™ chromatography–liquid chromatography–tandem mass spectrometry

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ABSTRACT

The work describes the development and validation of an analytical method for simultaneous determination of 58 pharmaceuticals and 19 metabolites and transformation products in environmental waters. The method is fully automated and consists of a direct injection of a small volume (several mL) of water sample to an on-line system composed of TurboFlow™ chromatography for the extraction and clean-up followed by liquid chromatography–electrospray-tandem mass spectrometry (TFC-LC–ESI-MS/MS). The feasibility and limitations of the technique in the analysis of environmental and wastewaters is discussed. The main advantages include high throughput, minimum sample manipulation, low error introduction, high selectivity, sensibility and reliability. The method provided a tool for the determination of a high number of active metabolites and transformation products and was successfully applied in the analysis of samples from the river Ebro basin.

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1. Introduction

Pharmaceuticals are detected in the environment in the ng L^{-1}–μg L^{-1} range [1], that may not pose much acute risk, however little is known about long-term effects and other receptors in non target organisms that could be sensitive [2]. Just a small portion (less than 10%) of the 3200 pharmaceuticals registered in Europe and North America have been measured in environmental samples. According to Howard and Muir [3] of 275 pharmaceuticals detected in the environment, 92 were rated as potentially bio-accumulative and 121 were rated as potentially persistent. Nevertheless, even those ones with relatively short environmental half-lives can be considered "pseudo-persistent" in the environment, due to their continual introduction into surface waters via wastewater treatment plant (WWTP) discharges [4]. Moreover, even though individual concentrations of any pharmaceutical compound might be low, the combined concentrations from compounds sharing a common mechanism of action could be substantial [2,5,6]. In addition to that, there must be taken into consideration that the compound may pass through several processes of transformation and degradation from the moment of intake, or even before, from its synthesis, until it arrives the environment. The term "degradation" here refers to the elimination of the parent compound without any knowledge whether the compound is mineralized or transformed in something else [7]. Products include both metabolites excreted via urine or faeces, and transformation products (TP) which can be formed by physico-chemical and biological processes in WWTPs or water works and/or in the environment, from parent pharmaceuticals and/or their metabolites released [8]. While substantial data exists regarding the occurrence and ecotoxicology of parent pharmaceuticals, much less is known about their metabolites and TP, whose existence is even unknown in most of cases. Very few pharmaceutical metabolites and TP are studied or monitored in the environment, so there is a need to strengthen the research in this area [8].

Therefore, the development of multi-residue analytical methods that will permit simultaneous monitoring of parent compounds and their metabolites and TPs is crucial to properly assess the risk posed by the presence of pharmaceuticals in the environment. This is of great importance as a synergistic effect of different pharmaceuticals on aquatic life might occur and has to be investigated [9]. Due to the very low levels of pharmaceuticals that may be present, an analyte pre-concentration procedure is compulsory in order to obtain the desired levels of analytical sensitivity, which often requires relatively large sample volumes (100–1000 mL) to be processed [10,11]. As awareness of more sustainable and

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environmentally friendly practices continues to grow, laboratories have also begun to look for ways to improve inefficient laboratory procedures and move towards "greener" alternative methods for environmental analysis. On-line sample pre-treatment is emerging as an effective approach with low or no solvent and chemical consumption [12] for the analysis of trace contaminants, such as drugs of abuse [13], pesticides [14] and pharmaceuticals [15] in water, or in other matrixes like food [16]. The development of on-line methods have additional clear advantages compared to off-line procedures, such as reduced sample manipulation (and subsequently lower probability of error introduction), smaller sample volume requirements (very welcome to relief storage problems) and, higher sample throughputs (and consequently an increase on the number of samples per unit of time that can be analyzed). All of which further improves the quality of the analytical data reported [17]. Most of the methods found in the literature for the analysis of pharmaceuticals in water, counts with liquid chromatography (LC) and detection by tandem mass spectrometry (MS/MS) [18–22].

The work presented here consists of a meticulous and successful development of a method for 58 pharmaceuticals and 19 metabolites/TPs, 8 of them with remaining pharmacologic activity, in environmental waters, by direct injection of small sample volumes (units of mL) on online TurboFlow™-LC–MS/MS. Up to authors knowledge this is the first time that on-line pre-treatment based on turbo-flow chromatography (TFC) is applied to environmental aqueous samples. A discussion about the feasibility of bidimensional chromatography, not only for the cleanup of that type of matrixes, but also for the pre-concentration of aqueous samples is included.

2. Material and methods

2.1. Chemicals

All pharmaceutical and TP standards were of high purity grade (>90%) and are listed in Supplementary data 1.

Both individual stock standard and isotopically labeled internal standard solutions were prepared on a weight basis in methanol (MeOH), except fluoroquinolones which were dissolved in a H_2O/MeOH mixture (1:1) containing 0.2% (v/v) hydrochloric acid (HCl), as they are slightly soluble in pure MeOH. After preparation, standards were stored at −20 °C. Stock solutions were renewed every 3 months. On the other hand, compounds with numbers (see Table 1) 69, 72, 73, and 74 were obtained as solutions in acetonitrile (ACN), 20 and 23 were dissolved in MeOH, at a concentration of 1 mg mL^{-1}, and 22 dissolved in a mixture MeOH/H_2O (1:1, v/v). A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in MeOH/H_2O (25:75, v/v). Working standard solutions, also prepared in MeOH/H_2O (25:75, v/v) mixture, were renewed before each analytical run. Working solutions were prepared in amber glass vials while standard mixtures were prepared in volumetric flasks wrapped with aluminum foil to prevent the exposure to light. A separate mixture of isotopically labeled internal standards, used for internal standard calibration, was prepared in MeOH and further dilutions also in MeOH/H_2O (25:75, v/v) mixture.

HPLC grade MeOH, ACN, acetone and H_2O, HCl 37%, trifluoro acetic acid (TFA) 99.8% and formic acid (thereon FA) 98% were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dehydrate (Na_2EDTA) was 99%, ammonium acetate (thereon NH_4Ac) was 98% and ammonium hydroxide (NH_4OH) was 28%, all of them from Sigma–Aldrich (Steinham, Germany). 2-Propanol 99.9% was obtained from Carlo Erba Reactifs (Val de Reuil, France).

2.2. Preparation of mobile phases

Several mobile phases were tested for the TFC extraction, as well as LC elution. The optimized ones are listed in Supplementary data 2. Six of them (a–f) were aqueous based, covering a large range of pH from 2 to 8, obtained by adjusting the pH of water with diluted solutions of NH_4OH. As organic based mobile phases, MeOH, ACN and mixtures of both were tested along with different proportions of weak acid modifiers.

2.3. Sample pre-treatment

The optimization of the method was carried out using spiked HPLC water, while optimized method is validated using groundwater (GW), river water (RW), influent waste water (IWW) and effluent waste water (EWW). Monitoring of river samples from Ebro River basin was carried out to demonstrate the applicability of the validated method. Amber glass bottles pre-rinsed with ultrapure water were used for sample collection. Due to the low sample volume requirements (7.5 mL), samples, even from extensive monitoring programs, can be easily stored in the freezer at −20 °C until they will be analyzed. In this fully automated methodology, sample handling is limited to a filtration step (performed to eliminate the particulate matter present in the samples), an addition of an acid/base modifier (0.1% FA + 0.02% TFA for the aliquot analyzed under positive electrospray ionization (ESI pos), or 10 mM NH_4Ac for the aliquot analyzed by negative electrospray ionization (ESI neg)) and 2% MeOH (to minimize the loss of the analytes through adsorption onto the surface of the glassware), and the fortification with a surrogate mixture at 100 ng L^{-1}. Regarding the filtration step, water samples were passed through 1 μm fiberglass filters from Whatman (Fairfield, CT, USA) followed by 0.45 μm nylon membrane filters from Teknokroma (Barcelona, Spain).

2.4. TFC clean up and LC separation

The online extraction and clean-up of the sample took place in a Thermo Scientific Transcend™ TLX system (Waltham, MA, USA), which consists of a CTC PAL Autosampler maintained at 10 °C, two quaternary high-pressure Accela pumps, one for online extraction (loading pump or TX pump) and one for analytical separation (eluting pump or LX pump), and 2 six-port switching valves controlled by Aria OS software. The system configuration is shown in Fig. 1. After its optimization, further explained in Sections 3.1 and 3.2, the validation and the determination of the real samples consisted of two sample injections, one of 2.5 mL in ESI pos mode and other of 5 mL in ESI neg mode. Thus, the total sample needed was 7.5 mL. The injected sample was passed through the TurboFlow™ column (TFC column) at a rate of 2 mL min^{-1}, by the loading mobile phase which was 0.1% FA + 0.02%TFA in ESI pos and 10 mM NH_4Ac in ESI neg run. The column used for the extraction (TFC column), were actually three columns connected in line, Cyclone P, C18-P XL and Cyclone MAX. All of them, with dimensions 1.0 mm × 50 mm, and purchased from Thermo Fisher Scientific (Waltham, MA, USA). During the phase of extraction, the switching valves were positioned as shown in Fig. 1A. TFC columns are filled with big particles of 30 μm. Thus, the interstitial space among them will be quite large and high flow rates will be able to be used keeping low back pressures. Those particles contain pores which are functionalized with different chemistries depending on the type of column. TurboFlow™ technology consists of two phenomena. The first one, a physical one, is inherent to the turbulent flow itself and consists of a diffusion phenomenon. Working at high flow rates, small molecules (<2000 Da) will diffuse into the pores, unlike the big ones (>8000 Da) which will not have enough time and will be flushed directly to the waste. The second mechanism is chemical and involves bonding to the

Table 1
Target compounds by therapeutical groups and used surrogates.

Therapeutic group	Number	Compound			Pharmacologic activity	CAS number	Corresponding surrogate
		Pharmaceutical	Transformation-product				
			Metabolite	Transformation product			
Analgesics/anti-inflammatories and metabolites (10)	1	Naproxen			Yes	22204-53-1	*Naproxen-d3*
	2	Indomethazine			Yes	53-86-1	*Indomethazine-d4*
	3	Diclofenac			Yes	15307-86-5	*Diclofenac-d4*
	4		4OH diclofenac		No	64118-84-9	*Tamoxifen-13C2*
	5	Mefenamic acid			Yes	61-68-7	*Mefenamic acid-d3*
	6	Acetaminophen			Yes	103-90-2	*Acetaminophen-d4*
	7		Salicylic acid		Yes	69-72-7	*Salicylic acid-d6*
	8	Propyphenazone			Yes	479-92-5	*Paroxetine-d4*
	9	Phenylbutazone			Yes	50-33-9	*Phenazone-d3*
	10	Phenazone			Yes	60-80-0	*Phenazone-d3*
Lipid regulators and metabolites (7)	11		Clofibric acid		Yes	882-09-7	*Clofibric acid-d4*
	12	Bezafibrate			Yes	41859-67-0	*Bezafibrate-d4*
	13	Fenofibrate			Yes	49562-28-9	*Fenofibrate-d6*
	14	Gemfibrozil			Yes	25812-30-0	*Gemfibrozil-d6*
	15	Pravastatin			Yes	81093-37-0	*Pravastatin-d3*
	16	Atorvastatin			Yes	134523-00-5	*Atorvastatin-d5*
	17		2OH atorvastatin		Yes	214217-86-4	*Flumequine-13C3*
Psychiatric drugs, metabolites and transformation products (12)	18	Paroxetine			Yes	61869-08-7	*Paroxetine-d4*
	19	Fluoxetine			Yes	54910-89-3	*Fluoxetine-d5*
	20	Diazepam			Yes	439-14-5	*Diazepam-d5*
	21		Desmethyl diazepam		Yes	1088-11-5	*Ofloxacin-d8*
	22		Oxazepam glucuronide		No	6801-81-6	*Diclofenac-d4*
	23	Lorazepam			Yes	846-49-1	*Diazepam-d5*
	24	Carbamazepine			Yes	298-46-4	*Carbamazepine-d10*
	25		2OH carbamazepine		No	68011-66-5	*Carbamazepine-d10*
	26		3OH carbamazepine		No	68011-67-6	*Phenazone-d3*
	27		10,11-Epoxi carbamazepine		Yes	36507-30-9	*Carbamazepine-d10*
	28			Acridone	No	578-95-0	*Carbamazepine-d10*
	29			Acridin	No	260-94-6	*Carbamazepine-d10*
Antihistaminics and metabolite (5)	30	Loratadine			Yes	79794-75-5	*Loratadine-d4*
	31	Famotidine			Yes	76824-35-6	*Famotidine-13C3*
	32	Ranitidine			Yes	66357-35-5	*Ranitidine-d6*
	33		Ranitidine N-oxide		No	73857-20-2	*Ranitidine-d6*
	34	Cimetidine			Yes	51481-61-9	*Cimetidine-d3*

Table 1 (*Continued*)

Therapeutic group	Number	Compound			Pharmacologic activity	CAS number	Corresponding surrogate
		Pharmaceutical	Transformation-product				
			Metabolite	Transformation product			
	35		Anhydroerythromycin		No	23893-13-2	*Atorvastatin-d5*
	36	Azithromycin			Yes	83905-01-5	*Azithromycin-d3*
	37	Roxithromycin			Yes	80214-83-1	*Loratadine-d4*
	38	Clarithromycin			Yes	81103-11-9	*Clarithromycin-d3*
	39	Josamycin			Yes	16846-24-5	*Loratadine-d4*
	40	Sulfamethoxazole			Yes	723-46-6	*Sulfamethoxazole-d4*
	41		N-acetyl sulfamethoxazole		No	21312-10-7	*Glyburide-d3*
	42	Sulfadiazine			Yes	68-35-9	*Sulfadiazine-d4*
	43		N-acetyl sulfadiazine		No	127-74-2	*Carbamazepine-d10*
Antibiotics and metabolites (20)	44	Sulfamethazine			Yes	57-68-1	*Sulfamethazine-d4*
	45		N-acetyl sulfamethazine		No	100-90-3	*Carbamazepine-d10*
	46	Ofloxacin			Yes	82419-36-1	*Ofloxacin-d8*
	47	Ciprofloxacin			Yes	85721-33-1	*Ciprofloxacin-d8*
	48	Norfloxacin			Yes	70458-96-7	*Norfloxacin-d5*
	49	Danofloxacin			Yes	112398-08-0	*Ofloxacin-d8*
	50	Enoxacin			Yes	74011-58-8	*Glyburide-d3*
	51	Flumequine			Yes	42835-25-6	*Flumequine-13C3*
	52	Trimethoprim			Yes	738-70-5	*Trimethoprim-d3*
	53	Chloramphenicol			Yes	56-75-7	*Diazepam-d5*
	54	Metronidazole			Yes	443-48-1	*Metronidazole-d4*
	55	Atenolol			Yes	29122-68-7	*Atenolol-d7*
	56	Sotalol			Yes	3930-20-9	*Sotalol-d6*
	57	Metoprolol			Yes	37350-58-6	*Metoprolol-d7*
	58	Propranolol			Yes	525-66-6	*Propranolol-d7*
	59		Propranolol-β-D-glucuronide		No	NA	*Flumequine-13C3*
	60	Timolol			Yes	26839-75-8	*Timolol-d5*
	61	Betaxolol			Yes	63659-18-7	*Propranolol-d7*
Cardiovascular drugs and metabolites (15)	62	Carazolol			Yes	57775-29-8	*Propranolol-d7*
	63	Pindolol			Yes	13523-86-9	*Pindolol-d7*
	64	Nadolol			Yes	42200-33-9	*Atenolol-d7*
	65	Enalapril			Yes	75847-73-3	*Enalapril-d5*
	66		Enalaprilat		Yes	84680-54-6	*Enalapril-d5*
	67	Hydrochlorothiazide			Yes	58-93-5	*Hydrochlorothiazide-d2*
	68	Lisinopril			Yes	83915-83-7	*Atenolol-d7*
	69	Furosemide			Yes	54-31-9	*Furosemide-d5*
	70	Albuterol			Yes	18559-94-9	*Albuterol-d3*
β-Agonists (2)	71	Clenbuterol			Yes	37148-27-9	*Clenbuterol-d9*
	72	Butalbital			Yes	77-26-9	*Clofibric acid-d4*
Barbiturates (3)	73	Pentobarbital			Yes	76-74-4	*Diclofenac-d4*
	74	Phenobarbital			Yes	50-06-6	*Phenobarbital-d5*
Antidiabetic and metabolite (2)	75	Glyburide			Yes	10238-21-8	*Glyburide-d3*
	76		Trans 4OH glyburide		Yes	23155-00-2	*Glyburide-d3*
Antineoplastic (1)	77	Tamoxifen			Yes	10540-29-1	*Tamoxifen-13C2*

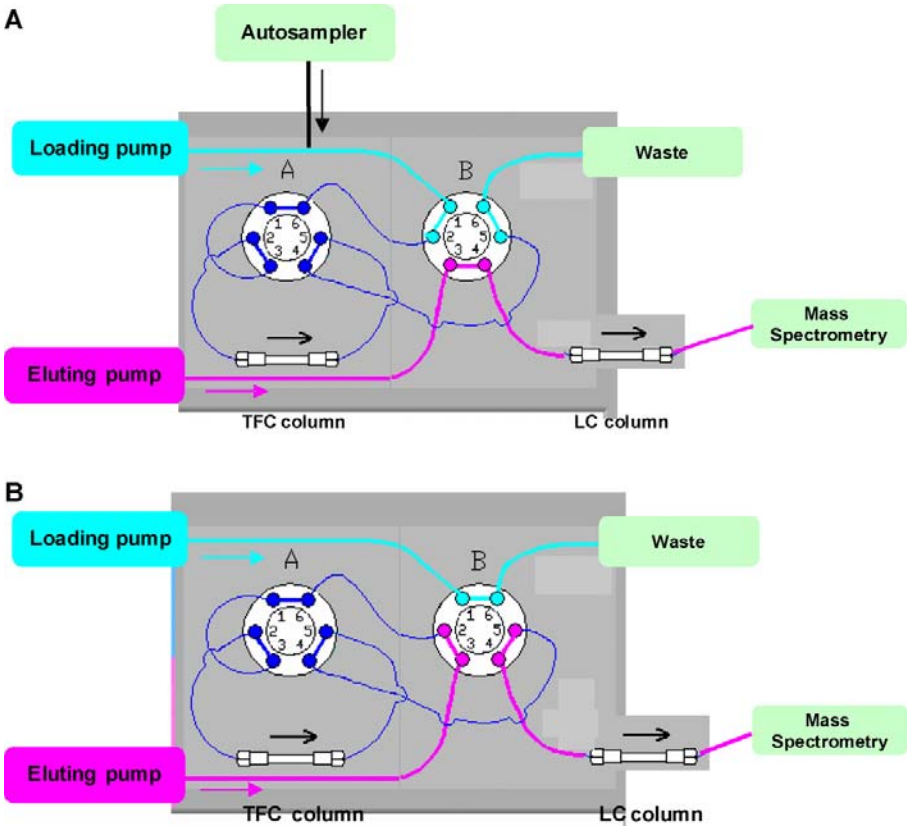

Fig. 1. Schematic set-up for the turbulent-flow chromatography (TFC).

functional groups. Therefore, only the small molecules with appropriate chemistry will keep retained inside the particles pores and bond to the functional groups. The rest will be flushed by the mobile phase going in and out the pores, and finally get to the waste [23].

Once all the sample has passed the TFC columns, and the extraction phase has concluded, the switching valves rotates and the system was configured in eluting position, as shown in Fig. 1B. The elution was carried out by the chromatographic mobile phases and gradient shown in Table 2. With it, the analytes were progressively eluted from the TFC column towards the analytical column (LC column), a Thermo Fisher Scientific Betasil Phenyl-Hexyl (50 mm × 3 mm, 3 μm). The total time of the analysis, including the extraction and the elution phases, was 22.42 and 21.48 min for ESI pos and neg, respectively.

2.5. Mass spectrometry

A Thermo Scientific TSQ Vantage™ triple quadrupole mass spectrometer with an ESI source (Waltham, MA, USA) was used as detector. For quantitative analysis, data acquisition was performed in selected reaction monitoring (SRM) mode, recording the transitions between the precursor ion and the two most abundant product ions for each target analyte. They are shown, along with the optimized compound-dependent S-lens RF Amplitude and collision energy (CE), in the Supplementary data 3. Resolution at first (Q1) and third quadrupole (Q3) was fixed at a peak width of 0.70. Settings for source-dependent parameters, common to both polarity modes, are as follows: a declustering collision energy of 10 V, a capillary temperature of 400 °C, a vaporizer temperature of 400 °C, a sheath gas pressure (N_2) of 0.6 L min^{-1}, an auxiliary gas pressure (N_2) of 4.5 L min^{-1} and an ion sweep gas pressure (N_2) of 0 L min^{-1}. Conversely, the ion spray voltage and the collision pressure, were, respectively, 4000 V and 1.0 mTorr in the ESI pos, and −3000 V and 1.2 mTorr in the ESI neg mode. Transitions were recorded in 2 min-windows around the retention time in order to achieve maximum sensitivity. Since window overlapping was taking place, a cycle time of 2 s was set to assure at least 12 points per peak. Instrument control and data acquisition and evaluation were performed with Thermo Fischer Scientific Xcalibur 2.1 software.

3. Results and discussion

3.1. Optimization of the bidimensional chromatography

In order to find the best conditions for the online TurboFlow™-LC, the protocol showed in Supplementary data 4 was followed. Two different runs, one with ESI pos and other with ESI neg were used. Thus, the extraction and chromatographic separation conditions were also optimized individually for each mode. Sensitivity and peak shape were the criteria followed to select each parameter. Only one parameter was optimized at the time, keeping the rest ones constant. Beginning with default conditions for the injection volume (20 μL) and loading mobile phase, the TFC column was the first parameter being optimized. 100% MeOH was used to elute complete and rapidly the analytes from the TFC column directly to the mass spectrometer. Several TFC columns were tested, i.e., Thermo Cyclone, Thermo Cyclone P, Thermo Cyclone MCX, Thermo C18, Thermo C18-P XL and Thermo Cyclone MAX, all of

Table 2
Chromatographic parameters used in the TFC and LC system.

(A) ESI pos

TFC system											LC system					
Step	Start time (min)	Seconds (s)	Flow rate (mL min^{-1})	Gradient	% A[a]	%B[h]	%C[c]	%D	SD	CD	Flow rate (mL min^{-1})	Gradient	%A[b]	%B[d]	%C[f]	%D[h]
1	0.00	150	2.00	Step	100	–	–	–	Load	←	0.50	Step	42.5	42.5	15	–
2	2.50	15	0.10	Step	–	100	–	–	Elute	→	0.50	Step	42.5	42.5	15	–
3	2.75	30	0.10	Step	–	100	–	–	Elute	→	0.50	Step	40	40	20	–
4	3.25	400	0.10	Step	–	100	–	–	Elute	→	0.60	Ramp	30	30	40	–
5	9.92	60	1.00	Step	–	100	–	–	Elute	→	0.75	Ramp	–	–	100	–
6	10.92	240	3.00	Step	–	100	–	–	Elute	→	0.75	Step	–	–	100	–
7	14.92	60	3.00	Step	–	100	–	–	Elute	→	1.00	Step	–	–	–	100
8	15.92	30	3.00	Step	–	100	–	–	Load	→	0.50	Step	–	–	100	–
9	16.42	30	3.00	Step	100	–	–	–	Load	→	0.50	Step	–	–	100	–
10	16.92	30	3.00	Step	–	100		–	Load	→	0.50	Ramp	42.5	42.5	15	–
11	17.42	30	3.00	Step	–		100	–	Load	→	0.50	Ramp	42.5	42.5	15	–
12	17.92	30	3.00	Step	–	100	–	–	Load	→	0.50	Ramp	42.5	42.5	15	–
13	18.42	240	2.00	Step	100	–	–	–	Load	←	0.50	Step	42.5	42.5	15	–

(B) ESI neg

TFC system											LC system					
Step	Start time (min)	Seconds (s)	Flow rate (mL min^{-1})	Gradient	%A[b]	%B[h]	%C[c]	%D[e]	SD	CD	Flow rate (mL min^{-1})	Gradient	%A	%B[g]	%C[b]	%D[h]
1	0.00	150	2.00	Step	–	–	100	–	Load	←	0.40	Step	–	45	55	–
2	2.50	15	0.10	Step	–	100	–	–	Load	→	0.40	Step	–	45	55	–
3	2.75	15	0.10	Step	–	–	–	100	Elute	→	0.40	Step	–	45	55	–
4	3.00	30	0.10	Step	–	–	–	100	Elute	→	0.40	Step	–	80	20	–
5	3.50	539	0.10	Step	–	100	–	–	Elute	→	0.40	Ramp	–	90	10	–
6	12.48	60	1.00	Step	100	–	–	–	Elute	→	0.40	Ramp	–	95	5	–
7	13.48	60	2.00	Step	–	100	–	–	Elute	→	0.40	Step	–	95	5	–
8	14.48	60	2.00	Step	–	100	–	–	Elute	→	0.40	Step	–	–	–	100
9	15.48	60	2.00	Step	–	100	–	–	Load	→	0.40	Step	–	95	5	–
10	16.48	60	2.00	Step	–	–	100	–	Load	→	0.40	Step	–	45	55	–
11	17.48	240	2.00	Step	–	–	100	–	Load	←	0.40	Step	–	45	55	–

a, b, c, d, e, f, g, h and i correspond with the mobile phases specified in Supplementary data 2. In brief:

[a] 0.1% FA + 0.02% TFA in water.
[b] 0.1% FA in water.
[c] 10 mM NH_4Ac in water.
[d] Water at pH 7.
[e] Water at pH 8.
[f] 0.3% FA + 0.02% TFA in ACN.
[g] 5 mM NH4Ac in MeOH:ACN (1:1, v/v).
[h] 0.1% FA in ACN/Acetone/Isopropanol (1:1:1, v/v/v).

them 0.5 mm × 50 mm. Thermo Cyclone P, Thermo C18-P XL and Thermo Cyclone MAX were the ones which extracted the highest amount of compounds. Then, the combination of two columns connected in series was tested and finally all three in series, being this last option the one that showed clearly the best sensitivity. The same trio of columns, but 1.0 mm × 50 mm, were also tested. The increase in the active surface resulted in better sensitivity. The next step was to optimize the loading mobile phase with which the injected sample is flushed through the TFC columns. In order to favor the extraction of the compounds, loading mobile phases covering a wide range of pH were tested. It was observed that the highest sensitivities in ESI pos were achieved with the acid solution (mobile phase (b) in Supplementary data 2). Regarding ESI neg, solution (d) with NH_4Ac and slightly basic pH was the one that provided the best sensitivity. In all following experiments, the sample injected contained the same percentage of modifiers as the loading mobile phase (for ESI pos: 0.1% FA + 0.02% TFA; for ESI neg: 10 mM NH_4Ac). Also 2% of MeOH are added, in order to minimize the loss of the analytes through adsorption onto the surface of the glassware. The influence of the loading flow rate on the extraction recovery was also tested. It was observed that the extraction efficiency decreased when the flow rate was 5 mL min^{-1} in comparison to 2 mL min^{-1}, so the latest was finally chosen. The next step was to increase the volume in order to get limits of detection (LODs) low enough to determine pharmaceuticals at environmentally relevant levels. On the other hand, the volume should be kept as low as possible in order to reduce the matrix effect. In addition, small sample sizes were preferred to relief problems of storage in the laboratories. Therefore, increasing volumes of 10, 100, 500, 1000, 2500 and 5000 μL were tested. The recovery of compounds is monitored and was observed that the efficiency of extraction was not influenced by the injection volume. Finally, for the run in ESI pos 2500 μL, and 5000 μL for the ESI neg were chosen.

The next step involved optimization of the chromatographic separation. Several analytical columns were tested, i.e., Thermo Hypersil PFP Gold (50 mm × 2.1 mm) and Thermo Betasil Phenyl-Hexyl (50 mm × 3 mm). Both, with 3 μm particle size. The latest was chosen in both ESI modes. It provided the best sensitivities. Different gradients were subsequently tested. Their selection and optimization was especially important because it had to perform an efficient elution of all the analytes from the TFC column to the LC one. In addition, a sufficient separation of all compounds with a good peak shape, including the separation of isomer metabolites of the hydroxy-carbamazepine was required. Different modifiers (FA, NH_4Ac, TFA) in different proportions were tested. Traces of TFA (0.02%) were found to improve the peak shape in ESI pos. It is worth to notice that the flow rate was also changing along the gradient in the ESI pos in order to shorten the run. In any case, in spite of such complex gradients, retention time was stable (see repeatability in Supplementary data 3).

With both phases of the bidimensional chromatography (extraction and separation) optimized, a check out of the carry over was carried out. It was observed that it was higher than 20% of LOQ for several compounds in ESI pos, as well as in ESI neg. The syringe used for the sample injection was found to be the main source of carry over, followed by the TFC column. To reduce the carry over, an exhaustive cleaning of the syringe after and before every injection was performed with the mobile phase used for the sample loading plus 2% MeOH (wash 1) and solvent (i) (wash 2) were incorporated. TFC column was also further cleaned with mobile phases (a), (d) and especially with (i) in ESI pos run (see steps 8, 9, 10, 11, 12 and 13), and with (d) and (i) in ESI neg run (see steps 10 and 11). The last steps in both runs (steps 13 and 11 in ESI pos and neg, respectively) were programmed to condition TFC and LC columns for the next injection and fill the loop with initial loading mobile phase. Finally, the optimized methods were 22.42 min long for the ESI pos run and 21.48 min for the ESI neg run. The characteristics of both runs (mobile phases, flow rates, run time, and gradient profiles of the TFC and LC systems) are summarized in Table 2.

3.2. Optimization of mass spectrometry detection

Selection of parent ions and optimum ionization mode for each compound was performed by infusing 2 mg L^{-1} individual standard solutions in MeOH, by full-scan mode at 0.1 and 0.7 of peak width resolution in Q1 and Q3, respectively. Of 77 compounds investigated, 61 substances (45 pharmaceuticals, 16 metabolites/TPs) showed higher response in ESI pos mode and 16 (13 pharmaceuticals and 3 metabolites/TPs) in ESI neg mode. In all cases, $[M+H]^+$ for ESI pos and $[M-H]^-$ for ESI neg were selected as parent ions. The best source-dependent parameters (spray voltage, vaporizer temperature, sheath gas pressure, ion sweep gas pressure, auxiliary gas pressure, capillary temperature and declustering voltage) were optimized for each compound, and subsequently a compromise was achieved to select the more appropriate one for ESI pos and ESI neg methods. The compound-dependent S-lens RF Amplitude was also optimized in full-scan mode, but at 0.7 peak width resolution in both Q1 and Q3, this time. Further identification of the two most abundant fragment ions and selection of the optimum CEs for each compound was carried out in the product ion scan mode, also by infusing standard solutions for each substance. The source-dependent collision pressure was tested in this second phase as well.

For quantitative analysis, the EU guidelines on quality assurance (EU Commission Decision 341 2002/657/EC) were met. Retention time and ratio SRM1/SRM2 are given in Supplementary data 3.

In order to increase the sensitivity, acquisition window was established individually for every transition. The shorter they were, better sensitivity was obtained. But at the same time, they had to be long enough to be trustworthy in case a change in the retention time took place. Finally, 2 min long individual windows for every transition were established both for ESI pos and ESI neg. Afterwards, cycle time was optimized. Cycle time is the time the detector devotes to scan all the transitions programmed per time unit, along the chromatogram. This is a key parameter, since a high cycle time would assure a sufficient number of registered points per peak to proceed with a precise quantification. But at the same time, the higher cycle time the lower sensitivity is achieved. Since acquisition windows were set along the chromatogram run, the number of cycles per time unit, and subsequently, the number of recorded points per peak, was changing along the chromatogram. Bearing all this in mind, a cycle time of 2 s was set, which assures the scanning of at least 12 points per peak for all the analytes, even for the ones with the narrowest peaks, but no further superfluous extra ones, which would decrease the sensitivity.

Representative SRM chromatograms obtained from the analysis of an aqueous standard mixture of analytes at a concentration of 500 ng L^{-1} versus a real river sample, applying the optimum method conditions are illustrated in Fig. 2.

3.3. Method validation

Once the method was developed and optimized in HPLC-grade water, it was validated for the analysis of environmental waters like GW and RW and for sewage water (IWW and EWW). The validation parameters tested were accuracy, sensitivity, matrix effect, precision and range of linearity. Unfavorable preliminary results were obtained for the validation parameters in complex wastewater matrices (EWW and IWW). That, along with other issues, resulted in conclusion that the method cannot be applied for wastewater samples, as it is further discussed in Section 3.4. On the other hand

Table 3
Validation parameters.

(A) Recoveries

Therapeutic group	Compound[a]	% Absolute recoveries			% Relative recoveries			% Signal suppression	
		HPLC	GW	SW	HPLC	GW	SW	GW	SW
Analgesics/anti-inflammatories and metabolites (10)	Naproxen	96.3	3.00	42.2	91.5	50.8	110	98.1	73.7
	Indomethazine	98.8	18.9	35.4	121	118	125	83.9	69.9
	Diclofenac	89.1	21.4	33.6	87.2	94.4	88.7	85.1	76.7
	4OH diclofenac	117	190	155	103	118	136	−10.7	−31.8
	Mefenamic acid	119	21.3	33.7	96.5	101	89.0	83.9	74.5
	Acetaminophen	25.6	25.6	25.4	101	109	158	−16.3	−94.0
	Salicylic acid	91.8	23.1	86.1	108	111	89.6	87.9	54.8
	Propyphenazone	115	156	94.2	105	102	105	−2.76	18.3
	Phenylbutazone	120	0.720	51.9	102	16.4	126	99.6	64.6
	Phenazone	115	106	42.9	101	103	104	22.7	62.7
Lipid regulators and metabolites (7)	**Clofibric acid**	91.9	12.5	22.4	87.0	88.8	107	86.3	75.6
	Bezafibrate	98.3	38.5	64.7	103	138	119	62.1	36.4
	Fenofibrate	88.4	114	47.6	109	103	59.2	19.6	46.2
	Gemfibrozil	89.4	14.3	29.2	92.7	99.1	144	84.0	67.3
	Pravastatin	96.8	31.2	45.9	98.9	111	84.1	71.3	57.8
	Atorvastatin	88.2	172	85.7	102	117	80.0	−38.7	−22.8
	2OH atorvastatin	124	220	253	51.1	68.8	108	−77.4	−440
Psychiatric drugs, metabolites and transformation products (12)	Paroxetine	103	162	72.6	99.1	106	86.4	−21.9	29.8
	Fluoxetine	93.6	162	78.8	102	113	97.5	−17.0	15.8
	Diazepam	105	167	85.7	101	102	90.3	−27.2	18.6
	Desmethyl diazepam	120	56.2	70.5	94.3	71.5	74.8	15.5	11.0
	Oxazepam glucuronide	57.3	26.8	42.9	81.0	127	113	67.4	47.8
	Lorazepam	117	179	118	107	109	124	−14.7	−0.890
	Carbamazepine	109	146	78.0	100	104	100	−11.4	28.2
	2OH carbamazepine	100	123	39.7	89.1	87.5	50.9	−22.7	54.2
	3OH carbamazepine	104	121	37.8	96.9	119	91.3	−16.3	60.7
	10,11-Epoxi carbamazepine	134	153	94.6	119	109	121	4.31	29.4
	Acridone	173	210	110	141	149	141	−3.50	36.5
	Acridin	135	140	40.5	116	99.3	52.0	13.8	70.0
Antihistaminics and metabolite (5)	Loratadine	97.7	190	78.1	102	108	86.4	−41.5	20.0
	Famotidine	99.0	82.5	26.2	103	82.5	100	16.7	64.2
	Ranitidine	83.2	77.9	5.33	100	97.3	86.9	5.12	92.0
	Ranitidine N-oxide	67.7	75.6	6.44	91.4	94.4	104	−14.3	88.5
	Cimetidine	109	200	21.2	100	102	83.6	−55.8	76.5
Antibiotics and metabolites (20)	**Anhydroerythromycin**	96.8	154	141	99.0	104	131	−38.8	−48.6
	Azithromycin	19.7	41.3	19.2	80.3	57.5	119	−115	−81.6
	Roxithromycin	77.0	128	55.5	58.6	72.6	61.4	−66.2	6.94
	Clarithromycin	85.4	179	78.2	69.7	117	86.9	−127	−20.8
	Josamycin	94.5	249	111	71.9	141	119	−163	−54.4
	Sulfamethoxazole	66.7	98.0	9.31	98.4	96.5	113	−75.2	82.4
	N-acetyl sulfamethoxazole	112	163	144	98.4	101	127	2.05	10.6
	Sulfadiazine	55.4	105	2.23	96.6	98.5	67.7	−135	94.3
	N-acetyl sulfadiazine	116	137	103	102	97.3	132	−3.15	11.3
	Sulfamethazine	52.9	93.8	3.34	102	89.4	92.1	−126	91.9
	N-acetyl sulfamethazine	100	131	116	98.0	93.0	149	−31.0	−4.90
	Ofloxacin	69.8	129	94.3	103	156	97.9	−84.6	−222
	Ciprofloxacin	71.5	119	88.2	100	69.5	87.2	−83.2	−300
	Norfloxacin	60.6	106	89.4	87.4	116	53.6	−75.5	−291
	Danofloxacin	49.9	122	130	79.5	148	135	−162	−373
	Enoxacin	118	176	121	103	109	106	−16.6	−3.28
	Flumequine	190	409	234	94.0	119	97.2	−36.2	−23.4
	Trimethoprim	97.9	162	41.0	93.0	110	142	−65.1	50.8
	Chloramphenicol	29.7	3.46	10.3	88.1	184	72.8	86.6	60.1
	Metronidazole	36.0	35.7	13.6	140.6	156	83.4	1.16	52.8
Cardiovascular drugs and metabolites (15)	Atenolol	101	130	20.6	99.8	110	86.7	−29.5	70.5
	Sotalol	87.8	118	44.1	102	85.8	69.7	−34.2	37.8
	Metoprolol	113	135	21.0	103	93.5	86.4	−7.81	81.3
	Propranolol	112	137	59.8	97.6	104	96.0	1.20	46.4
	Propranolol-β-D-glucuronide	139	380	26	107	127	121	−53.8	−90.5
	Timolol	103	122	18.6	99.6	101	88.4	−6.13	81.9
	Betaxolol	108	146	74.5	100	110	120	8.47	3.19
	Carazolol	114	121	55.5	100	91.3	89.2	10.7	51.5
	Pindolol	102	0.310	1.45	101	116	92.8	99.8	98.6
	Nadolol	102	109	15.5	108	91.8	65.0	−5.94	82.2
	Enalapril	101	132	70.5	100	102	77.3	−22.3	19.2
	Enalaprilat	97.1	128	93.4	105	121	134	−1.23	−4.09
	Hydrochlorothiazide	145	14.9	5.59	112	98.9	12.0	95.3	98.3
	Lisinopril	97.2	103	83.0	101	87.4	345	−8.02	−16.4
	Furosemide	69.8	39.7	87.0	88.2	84.3	109	55.1	1.49

Table 3 (*Continued*)

(A) Recoveries

Therapeutic group	Compound[a]	% Absolute recoveries			% Relative recoveries			% Signal suppression	
		HPLC	GW	SW	HPLC	GW	SW	GW	SW
β-Agonists (2)	Albuterol	72.5	53.2	3.11	100	108	91.9	20.4	92.7
	Clenbuterol	94.4	114	10.8	84.0	87.6	84.6	2.28	88.6
Barbiturates (3)	Butalbital	62.0	19.3	26.6	97.8	137	124	71.5	60.9
	Pentobarbital	74.6	20.5	31.6	115	97.5	83.3	72.5	57.7
	Phenobarbital	68.0	31.7	43.2	109	113	79.2	76.2	67.5
Antidiabetic and metabolite (2)	Glyburide	144	186.9	117.96	105	116	103	−6.88	18.3
	Trans 4OH glyburide	187	107.4	63.5	115	66.5	55.7	53.0	75.3
Antineoplastic (1)	Tamoxifen	76.1	63.3	2.02	102	135	86.5	16.8	93.5

(B) Limit of detection and quantification

Therapeutic group	Compound[a]	LOD instrumental (pg)	LOD ($ng\,L^{-1}$)			LOQ instrumental (pg)	LOQ ($ng\,L^{-1}$)		
			HPLC	GW	SW		HPLC	GW	SW
Analgesics/anti-inflammatories and metabolites (10)	Naproxen	1.09	2.66	21.5	19.0	3.63	8.88	71.8	63.4
	Indomethazine	1.17	0.870	0.460	0.180	3.90	2.90	1.53	0.600
	Diclofenac	1.23	0.560	18.1	10.2	4.11	3.51	60.4	34.0
	4OH diclofenac	2.40	0.120	1.65	0.280	7.99	0.390	5.49	0.920
	Mefenamic acid	0.990	0.940	2.47	2.84	3.30	3.13	8.24	9.48
	Acetaminophen	6.81	19.9	21.7	14.5	22.7	66.3	72.2	48.3
	Salicylic acid	4.14	13.6	12.6	5.64	13.8	49.9	42.1	18.8
	Propyphenazone	1.09	0.360	0.960	0.0300	3.65	1.19	3.19	0.100
	Phenylbutazone	4.39	1.15	3.58	14.2	14.6	3.83	11.9	47.4
	Phenazone	1.47	0.600	1.11	2.62	4.90	2.00	3.70	8.74
Lipid regulators and metabolites (7)	**Clofibric acid**	3.26	1.63	4.18	2.70	10.9	5.43	13.9	9.00
	Bezafibrate	0.0700	0.470	1.74	0.192	0.250	1.55	5.81	0.640
	Fenofibrate	5.92	1.36	2.05	1.61	19.7	4.52	6.83	5.35
	Gemfibrozil	15.70	4.04	11.8	19.8	52.3	13.5	39.2	66.1
	Pravastatin	0.440	0.470	3.97	7.71	1.47	1.40	13.2	25.7
	Atorvastatin	0.240	0.100	0.170	0.0700	0.810	0.340	0.560	0.220
	2OH atorvastatin	0.940	0.0400	0.640	0.610	3.13	0.130	2.12	2.02
Psychiatric drugs, metabolites and transformation products (12)	Paroxetine	1.11	1.21	4.83	4.55	3.71	4.02	16.1	15.2
	Fluoxetine	2.34	2.55	6.57	3.74	7.81	8.51	21.9	12.5
	Diazepam	1.77	1.28	1.46	1.06	5.90	4.25	4.86	3.55
	Desmethyl diazepam	0.190	0.480	0.850	0.0800	0.640	1.59	2.85	0.280
	Oxazepam glucuronide	0.480	0.0400	10.5	13.3	1.60	0.110	35.0	44.3
	Lorazepam	4.63	1.76	2.13	2.80	15.4	5.86	7.09	9.32
	Carbamazepine	0.250	0.0900	0.320	0.135	0.830	0.280	1.06	0.450
	2OH carbamazepine	1.32	1.86	4.62	17.9	4.39	6.21	15.4	59.8
	3OH carbamazepine	0.900	0.210	0.440	0.210	2.99	0.700	1.48	0.700
	10,11-Epoxi carbamazepine	9.67	3.80	6.80	4.04	32.2	12.7	22.7	13.5
	Acridone	1.11	0.680	0.900	1.16	3.70	2.27	3.01	3.88
	Acridin	0.610	0.550	1.41	0.860	2.04	1.84	4.69	2.86
Antihistaminics and metabolite (5)	Loratadine	0.880	1.11	1.19	1.43	2.94	3.72	3.96	4.78
	Famotidine	0.500	0.950	3.91	2.85	1.66	3.16	13.0	9.50
	Ranitidine	0.230	0.570	1.07	2.87	0.780	1.91	3.56	9.55
	Ranitidine N-oxide	0.710	3.63	4.52	4.32	2.38	12.1	15.0	14.4
	Cimetidine	0.340	0.400	1.05	1.09	1.14	1.33	3.50	3.64
Antibiotics and metabolites (20)	**Anhydroerythromycin**	0.0100	0.0100	5.72	7.03	0.0400	0.0300	19.1	23.4
	Azithromycin	0.970	5.47	3.95	3.18	3.23	18.2	13.2	10.6
	Roxithromycin	1.67	0.630	4.53	0.260	5.57	2.08	15.1	0.870
	Clarithromycin	3.55	1.30	3.16	4.89	11.8	4.32	10.5	16.3
	Josamycin	0.180	0.0200	12.3	0.0800	0.590	0.0800	41.0	0.270
	Sulfamethoxazole	2.28	0.480	3.16	6.84	7.59	1.61	10.5	22.8
	N-acetyl sulfamethoxazole	0.0700	0.0800	0.0100	0.0500	0.220	0.260	0.0500	0.160
	Sulfadiazine	3.08	1.48	2.62	5.67	10.3	4.95	8.75	18.9
	N-acetyl sulfadiazine	0.0400	0.0400	2.76	9.95	0.130	0.150	9.21	33.1
	Sulfamethazine	4.07	1.83	4.38	49.3	13.6	6.10	14.6	164
	N-acetyl sulfamethazine	0.240	0.0500	0.140	1.38	0.810	0.150	0.460	4.60
	Ofloxacin	5.95	0.0200	23.8	29.1	19.8	0.0600	79.4	96.8
	Ciprofloxacin	6.87	9.95	11.0	7.03	22.9	33.2	36.7	23.4
	Norfloxacin	6.97	19.4	16.0	17.3	23.2	64.8	53.4	57.8
	Danofloxacin	0.110	1.05	5.76	4.45	0.350	3.51	19.2	14.8
	Enoxacin	5.32	1.25	2.38	1.22	17.8	4.18	7.94	4.06
	Flumequine	5.66	1.75	2.33	1.04	18.9	5.82	7.78	3.45
	Trimethoprim	0.380	0.200	0.350	0.0390	1.25	0.660	1.17	0.130
	Chloramphenicol	0.430	1.61	3.99	1.95	1.45	5.36	13.3	6.49
	Metronidazole	5.46	11.1	17.5	19.1	18.2	37.2	58.5	63.6

Table 3 (*Continued*)

(B) Limit of detection and quantification

Therapeutic group	Compound[a]	LOD instrumental (pg)	LOD (ng L^{-1})			LOQ instrumental (pg)	LOQ (ng L^{-1})		
			HPLC	GW	SW		HPLC	GW	SW
Cardiovascular drugs and metabolites (15)	Atenolol	0.700	0.380	2.17	1.87	2.32	1.26	7.23	6.23
	Sotalol	2.24	6.46	4.31	2.52	7.47	21.5	14.4	8.39
	Metoprolol	1.60	0.570	0.730	4.15	5.35	1.90	2.44	13.8
	Propranolol	0.920	1.23	2.86	1.44	3.06	4.08	9.53	4.79
	Propranolol-β-D-glucuronide	1.04	0.670	11.4	12.3	3.46	2.23	38.0	40.9
	Timolol	0.530	0.150	0.780	0.430	1.76	0.510	2.59	1.42
	Betaxolol	0.830	0.650	0.990	0.710	2.76	2.15	3.31	2.36
	Carazolol	0.370	0.720	6.34	2.34	1.25	2.41	21.1	7.82
	Pindolol	0.200	0.250	23.7	4.58	0.670	0.820	79.0	15.2
	Nadolol	0.200	0.150	0.430	0.120	0.660	0.510	1.44	0.410
	Enalapril	0.450	1.12	2.68	1.99	1.52	3.74	8.93	6.62
	Enalaprilat	3.55	1.19	3.25	1.68	11.8	3.97	10.8	5.60
	Hydrochlorothiazide	0.490	1.62	1.26	6.81	1.63	5.41	4.21	22.7
	Lisinopril	0.790	0.870	3.14	3.95	2.65	2.90	10.5	13.2
	Furosemide	0.440	5.85	28.2	8.14	1.46	19.5	94.2	27.1
β-Agonists (2)	Albuterol	0.0900	0.0400	0.130	0.800	0.30	0.140	0.430	2.67
	Clenbuterol	0.600	0.540	1.48	2.28	2.01	1.79	4.95	7.61
Barbiturates (3)	Butalbital	0.470	0.340	1.43	28.2	1.58	1.15	4.77	94.0
	Pentobarbital	1.72	1.07	2.86	20.1	5.72	3.57	9.54	66.9
	Phenobarbital	1.15	2.06	0.690	16.7	3.83	8.71	2.30	55.5
Antidiabetic and metabolite (2)	Glyburide	0.900	0.100	0.800	0.260	3.00	0.350	2.66	0.860
	Trans 4OH glyburide	0.240	5.98	26.1	5.06	0.820	19.9	87.0	16.9
Antineoplastic (1)	Tamoxifen	0.0800	0.0400	0.110	0.380	0.260	0.130	0.350	1.27

(C) Repeatability and linearity

Therapeutic group	Compound[a]	Repeatability (%RSD)		Linearity	
		Intraday	Interday	R^2	Range (ng L^{-1})
Analgesics/anti-inflammatories and metabolites (10)	Naproxen	17.5	42.2	0.9992	LOQ-5000
	Indomethazine	1.47	2.86	0.9997	LOQ-5000
	Diclofenac	25.0	0.890	0.9960	LOQ-500
	4OH diclofenac	6.85	3.70	0.9989	LOQ-500
	Mefenamic acid	19.8	0.440	0.9983	LOQ-500
	Acetaminophen	10.8	6.44	0.9996	LOQ-5000
	Salicylic acid	32.0	1.58	0.9913	LOQ-5000
	Propyphenazone	0.560	1.78	1.0000	LOQ-5000
	Phenylbutazone	4.08	19.2	0.9995	LOQ-5000
	Phenazone	0.300	3.46	1.0000	LOQ-5000
Lipid regulators and metabolites (7)	**Clofibric acid**	27.2	3.80	0.9999	LOQ-5000
	Bezafibrate	18.2	2.61	0.9997	LOQ-5000
	Fenofibrate	8.91	40.6	0.9999	LOQ-5000
	Gemfibrozil	12.7	6.27	0.9992	LOQ-5000
	Pravastatin	16.9	0.940	1.0000	LOQ-5000
	Atorvastatin	0.720	8.71	0.9999	LOQ-5000
	2OH atorvastatin	8.88	8.73	0.9995	LOQ-5000
Psychiatric drugs, metabolites and transformation products (12)	Paroxetine	1.39	6.77	1.0000	LOQ-5000
	Fluoxetine	9.12	7.30	1.0000	LOQ-5000
	Diazepam	3.10	6.26	1.0000	LOQ-5000
	Desmethyl diazepam	11.4	7.86	1.0000	LOQ-5000
	Oxazepam glucuronide	1.23	2.43	0.9995	LOQ-500
	Lorazepam	0.600	6.51	0.9999	LOQ-5000
	Carbamazepine	7.19	5.82	0.9998	LOQ-5000
	2OH carbamazepine	12.9	6.62	0.9990	LOQ-5000
	3OH carbamazepine	7.73	7.82	0.9980	LOQ-5000
	10,11-Epoxi carbamazepine	15.6	25.0	1.0000	LOQ-5000
	Acridone	0.00472	0.740	0.9998	LOQ-5000
	Acridin	1.57	1.77	0.9999	LOQ-5000
Antihistaminics and metabolite (5)	Loratadine	6.58	7.63	0.9994	LOQ-5000
	Famotidine	2.60	7.97	0.9988	LOQ-5000
	Ranitidine	0.910	12.1	1.0000	LOQ-5000
	Ranitidine N-oxide	1.62	9.76	0.9999	LOQ-5000
	Cimetidine	3.31	7.44	0.9989	LOQ-5000

Table 3 (*Continued*)

(C) Repeatability and linearity

Therapeutic group	Compound[a]	Repeatability (%RSD)		Linearity	
		Intraday	Interday	R^2	Range (ng L^{-1})
	Anhydroerythromycin	5.25	2.49	1.0000	LOQ-5000
	Azithromycin	1.69	7.61	0.9937	LOQ-5000
	Roxithromycin	0.690	6.12	0.9997	LOQ-5000
	Clarithromycin	5.68	10.6	1.0000	LOQ-5000
	Josamycin	0.100	13.4	0.9998	LOQ-5000
	Sulfamethoxazole	12.4	5.53	0.9998	LOQ-5000
	N-acetyl sulfamethoxazole	9.77	10.0	0.9998	LOQ-5000
	Sulfadiazine	14.8	11.4	0.9979	LOQ-5000
	N-acetyl sulfadiazine	1.74	12.2	0.9997	LOQ-5000
Antibiotics and metabolites	Sulfamethazine	6.83	16.8	1.0000	LOQ-5000
(20)	**N-acetyl sulfamethazine**	12.2	6.36	0.9996	LOQ-5000
	Ofloxacin	2.08	9.08	0.9994	LOQ-5000
	Ciprofloxacin	6.65	4.36	1.0000	LOQ-5000
	Norfloxacin	0.440	28.1	0.9994	LOQ-5000
	Danofloxacin	6.51	23.4	0.9984	LOQ-5000
	Enoxacin	2.26	3.79	0.9998	LOQ-5000
	Flumequine	0.440	4.91	0.9999	LOQ-5000
	Trimethoprim	3.87	5.24	1.0000	LOQ-5000
	Chloramphenicol	0.690	2.25	0.9994	LOQ-500
	Metronidazole	2.11	8.18	0.9936	LOQ-5000
	Atenolol	5.88	6.03	1.0000	LOQ-5000
	Sotalol	2.21	3.48	1.0000	LOQ-5000
	Metoprolol	4.83	7.57	0.9999	LOQ-5000
	Propranolol	5.08	7.46	1.0000	LOQ-5000
	Propranolol-β-D-glucuronide	17.9	6.42	0.9950	LOQ-5000
	Timolol	5.63	9.59	1.0000	LOQ-5000
	Betaxolol	31.0	10.1	1.0000	LOQ-5000
Cardiovascular drugs and metabolites (15)	Carazolol	4.64	7.83	0.9999	LOQ-5000
	Pindolol	3.37	8.17	0.9947	LOQ-5000
	Nadolol	2.06	8.20	0.9999	LOQ-5000
	Enalapril	1.33	2.29	1.0000	LOQ-5000
	Enalaprilat	4.01	5.83	1.0000	LOQ-5000
	Hydrochlorothiazide	0.160	3.04	0.9972	LOQ-500
	Lisinopril	1.18	4.20	0.9996	LOQ-5000
	Furosemide	8.05	5.81	0.9942	LOQ-5000
β-Agonists (2)	Albuterol	5.85	7.25	1.0000	LOQ-5000
	Clenbuterol	6.93	5.47	0.9999	LOQ-5000
	Butalbital	3.94	8.09	1.0000	LOQ-5000
Barbiturates (3)	Pentobarbital	10.7	3.42	0.9984	LOQ-500
	Phenobarbital	7.97	11.4	0.9998	LOQ-5000
Antidiabetic and	Glyburide	1.65	9.47	0.9998	LOQ-5000
metabolite (2)	**Trans 4OH glyburide**	14.1	3.88	0.9950	LOQ-5000
Antineoplastic (1)	Tamoxifen	2.67	8.93	0.9982	LOQ-5000

[a] In bold the 19 transformation-products and with regular format, the 58 pharmaceuticals.

satisfactory results are obtained for GW and RW and complete numerical data are given in Table 3.

3.3.1. Accuracy

Absolute recoveries for target compounds were determined for all different matrixes by spiking samples ($n = 3$) at two levels of concentration, i.e., 20 ng L^{-1} and 100 ng L^{-1} for HPLC-grade water, GW and RW, and 50 ng L^{-1} and 500 ng L^{-1} for both IWW and EWW. Those levels were chosen as typical low and high concentrations for most of compounds in those types of waters. For each type of matrix, recoveries were determined as percentages by comparing the peak areas obtained after the whole optimized method (on-line method) with the peak areas obtained from direct injection (20 μL) of equivalent amounts of standards on the LC column (off-line method). In the off-line method, the analytical column and the chromatographic gradient were the same ones optimized for the on-line method, and the standards were dissolved in the mixture of solvents at the initial chromatographic conditions. As real samples (GW, RW, EWW and IWW) may already contain target compounds, non-spiked matrix samples were analyzed and the peak areas were afterwards subtracted. Relative recoveries were determined as the ratio between the absolute recoveries for each compound versus the ones for the corresponding surrogate.

As can be observed in Table 3A, absolute recoveries in HPLC-grade water were between 50 and 150% for all compounds, except for 6 of them (acetaminophen, acridone, azithromycin, flumequine, chloramphenicol, and metronidazole). This shows that the TFC was applied successfully as extraction method. In fact, it worked better than the on-line SPE for the same compounds as published previously [15], where only 70% of the compounds showed an absolute recovery in that range for HPLC-grade water. The absolute recovery got worse with the presence of matrix, with recoveries between 50 and 150% for less of half of the compounds (45 and 48% for GW and RW, respectively). That is a consequence of the matrix effect, which is discussed further in this section. However, this situation was corrected by the use of surrogates. Thus, the total of compounds in HPLC-grade and almost all of them in GW and RW (95 and 96%, respectively) showed a relative recovery between 50 and 150%. This confirms that the use of isotopically labeled compounds as surrogate is essential to correct for potential losses during sample manipulation and extraction, as well as for matrix effects. In the present work, 46 isotopically labeled surrogates were used. That means that the method is near to the isotopic dilution method, with more two thirds of the compounds having a specific isotopically labeled surrogate. Nevertheless, for the compounds with no specific surrogate, the most appropriate was selected among the ones

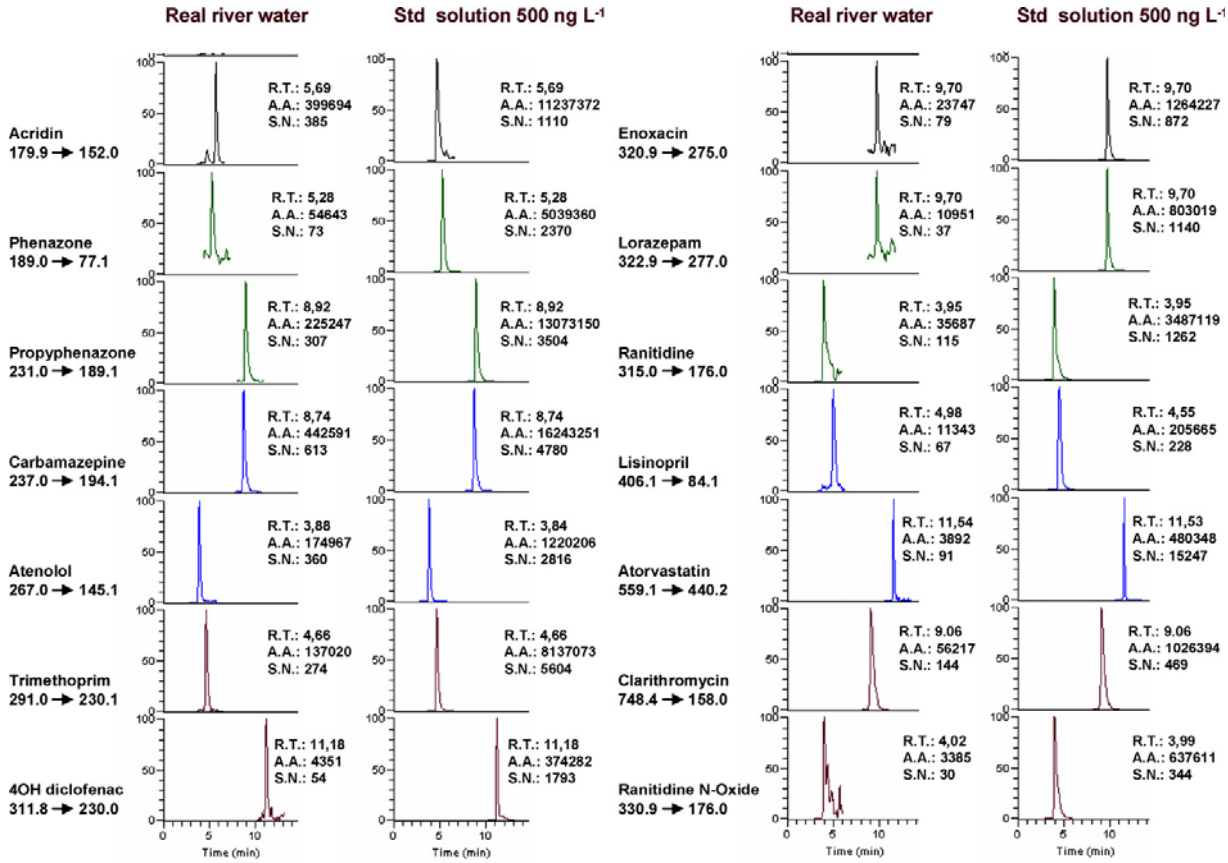

Fig. 2. Chromatogram of a standard solution in HPLC-grade water at 500 ng L^{-1} versus a real river sample, for some of the compounds detected.

available by testing how good they were in correcting the losses in the extraction recovery. The assignment is shown in Table 1. It is worth to mention that with on-line technologies that correction for extraction recoveries is automatically performed because both the samples and aqueous standards are processed through exactly the same analytical procedure.

3.3.2. Matrix effect

The matrix effect was evaluated for each compound by comparing the peak area obtained for GW and RW spiked with the analytes at 100 ng L^{-1} (after subtraction of the peak areas corresponding to the native analytes present in the sample) with those obtained from HPLC-grade water solutions spiked at the same concentration. In the absence of matrix effects, the analytes' peak areas should be similar in both types of matrices, whereas in the presence of matrix effects, the ones from GW and RW are greater or lower than from HPLC-grade ones, depending on whether there is signal enhancement or suppression, respectively. These effects were quantified according with the following equation:

$$\text{Signal suppression}\,(\%) = 100 - \left(\frac{(\text{Area}_{\text{sm}} - \text{Area}_{\text{m}}) \times 100}{\text{Area}_{\text{HPLC}}}\right)$$

where Area_{sm} is the analyte peak area in the spiked matrix sample (GW, RW), Area_{m} is the analyte peak are in the nonspiked matrix (if any), and $\text{Area}_{\text{HPLC}}$ is the analyte peak are in the spiked HPLC water sample (see Table 3A for all the values). In GW, around half of the compounds showed signal suppression and the other half signal enhancement (matrix effect values in negative). Unlikely, in the RW there was a clear tendency towards signal suppression. Thus, 75% of compounds showed a positive suppression effect and 10 of them, the most polar ones, showed the values higher than 80%. This correlation between the polarity and the suppression effect was previously observed by Postigo et al. [13]. Consequently, the quantitation by the external standard method would have led to inaccurate results. However, with the present methodology, the varying matrix effects and recoveries observed are compensated through the use of surrogates for quantitation, with the already mentioned automatic correction.

3.3.3. Sensitive

The limits of detection (LODs) and quantification (LOQs) of the present method were experimentally determined as the concentration of analyte giving a signal-to-noise ratio of 3 and 10, respectively. Table 3B shows the LODs and LOQs calculated for GW, RW and HPLC-grade water. As expected, the LODs and LOQs were increasing from HPLC-grade water to GW and to RW. This is due to the matrix effect along with the higher background noise, combined with insufficient detector selectivity. Nevertheless, for the 73% of compounds, LODs were lower than 5 ng L^{-1} in GW. In RW, LODs were under 10 ng L^{-1} for the 75% of analytes. This means that the method counts with sensitivities sufficient for the analysis of pharmaceuticals in this type of samples. In addition, it is worth to remark that, to achieve that level of method sensitivity with a regular off-line procedure (where, for instance, the sample extract is

Table 4
Concentrations found and mean value in ng L^{-1} for the compounds detected at least in one of the samples in river samples in the Ebro basin (NE Spain).

Therapeutic group	Compound[a]	Downstream Gasteiz	Downstream Logroño	Downstream Pamplona	Downstream Tudela	Downstream Zaragoza	Downstream Lleida
Analgesics/anti-inflammatories and metabolites	Naproxen	n.d.[b]	n.d.	bloq.[c]	bloq.	bloq.	63.5
	Indomethazine	bloq.	n.d.	n.d.	n.d.	n.d.	n.d.
	Diclofenac	bloq.	n.d.	260	n.d.	bloq.	n.d.
	4OH diclofenac	39.8	bloq.	48.2	2.37	7.76	n.d.
	Acetaminophen	n.d.	n.d.	n.d.	n.d.	156	n.d.
	Salicylic acid	59.8	26.2	31.9	18.8	37.4	24.9
	Propyphenazone	34.7	2.26	10.0	1.49	1.55	2.92
	Phenazone	37.5	n.d.	18.6	n.d.	bloq.	n.d.
Lipid regulators and metabolites	**Clofibric acid**	bloq.	n.d.	n.d.	n.d.	n.d.	n.d.
	Bezafibrate	51.3	n.d.	bloq.	bloq.	2.97	bloq.
	Gemfibrozil	113	n.d.	69.7	n.d.	60.4	71.20
	Pravastatin	n.d.	n.d.	7.730	n.d.	0.07	n.d.
Psychiatric drugs, metabolites and transformation products	Paroxetine	7.73	n.d.	n.d.	7.28	n.d.	7.76
	Fluoxetine	14.5	n.d.	14.3	13.9	14.0	n.d.
	Desmethyl diazepam	n.d.	n.d.	3.99	n.d.	n.d.	n.d.
	Oxazepam glucuronide	70.8	39.4	bloq.	n.d.	bloq.	bloq.
	Lorazepam	45.7	n.d.	50.2	17.1	17.4	18.1
	Carbamazepine	90.4	9.80	34.4	7.91	7.59	9.33
	2OH carbamazepine	61.7	bloq.	22.8	2.56	3.53	3.73
	3OH carbamazepine	70.0	2.99	25.3	1.36	4.57	5.48
	10,11-Epoxi carbamazepine	1670	181	673	114	81.6	126
	Acridin	16.8	n.d.	11.4	9.64	bloq.	10.3
Antihistaminics and metabolites	Loratadine	n.d.	n.d.	17.1	n.d.	3.96	n.d.
	Ranitidine	104	34.6	41.8	3.99	6.24	6.44
	Ranitidine N-oxide	78.4	32.3	21.5	n.d.	n.d.	n.d.
	Anhydroerythromycin	0.96	n.d.	n.d.	n.d.	n.d.	n.d.
Antibiotics and metabolites	Azithromycin	n.d.	41.1	bloq.	bloq.	bloq.	bloq.
	Clarithromycin	141	13.1	45.4	12.8	17.2	28.0
	Sulfamethoxazole	bloq.	n.d.	17.2	0.10	n.d.	n.d.
	N-acetyl sulfamethoxazole	4.41	n.d.	n.d.	n.d.	n.d.	n.d.
	Sulfadiazine	51.4	44.8	42.7	41.0	51.0	43.4
	Sulfamethazine	n.d.	8.28	64.8	5.50	n.d.	bloq.
	Ofloxacin	bloq.	n.d.	bloq.	n.d.	bloq.	bloq.
	Ciprofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	bloq.
	Danofloxacin	n.d.	n.d.	bloq.	n.d.	n.d.	n.d.
	Enoxacin	bloq.	n.d.	bloq.	n.d.	n.d.	n.d.
	Flumequine	n.d.	n.d.	n.d.	bloq.	n.d.	n.d.
	Trimethoprim	59.9	4.89	26.1	10.3	12.0	7.12
	Chloramphenicol	bloq.	bloq.	n.d.	n.d.	0	n.d.
Cardiovascular drugs and metabolites	Atenolol	475	1.36	92.4	n.d.	11.2	10.8
	Sotalol	bloq.	bloq.	bloq.	bloq.	n.d.	n.d.
	Metoprolol	14.8	6.32	7.30	6.22	6.45	6.75
	Propranolol	18.8	9.89	13.5	8.95	9.56	9.92
	Timolol	n.d.	3.15	3.630	bloq.	3.28	bloq.
	Carazolol	8.04	8.17	7.960	bloq.	7.85	7.99
	Pindolol	bloq.	7.65	n.d.	n.d.	n.d.	bloq.
	Enalapril	2.20	n.d.	2.180	1.43	2.91	2.30
	Enalaprilat	n.d.	8.78	11.6	9.36	bloq.	bloq.
	Hydrochlorothiazide	494	147	410	68.9	190	253
	Lisinopril	116	n.d.	n.d.	19.1	n.d.	21.3
	Furosemide	108	n.d.	bloq.	n.d.	bloq.	n.d.
β-Agonists	Albuterol	1.43	n.d.	n.d.	n.d.	n.d.	n.d.
Barbiturates	Phenobarbital	99.5	n.d.	78.1	n.d.	31.91	bloq.
Antineoplastic	Tamoxifen	22.8	22.7	22.6	22.4	22.4	22.5

[a] Metabolites and Transformation-products in bold.
[b] n.d., not detected.
[c] bloq., below the limit of quantification.

reduced to 1 mL, from which 20 μL is injected in the LC–MS/MS system) the volume of sample to be extracted would have to be higher than 125 mL, versus units of mL of sample in the present method (2.5 and 5 mL, for ESI pos and neg, respectively). Values of LODs and LOQs presented here are similar to the ones reported previously for off-line [24,25], and online SPE methods [15].

3.3.4. Precision

The overall method repeatability, calculated as the relative standard deviation (RSD) of the replicate ($n = 5$) analysis of HPLC-grade water spiked with a standard of mixture of the analytes (100 ng L^{-1}), was satisfactory, with RSD values lower than 20% for most of the compounds in analysis made during the same day (intraday) and also comparing different days (interday), see Table 3C. The repeatability of results is one of the main advantages of automated online method and is because manipulation of the samples is almost avoided.

3.3.5. Linearity

Six point calibration curve was constructed, using least-squares linear regression analysis, from application of the overall method to HPLC-grade water spiked with the analytes at concentrations ranging from 0.05 ng L^{-1} (or the limit of quantification of higher) to 5000 ng L^{-1}. Only for diclofenac and its metabolite

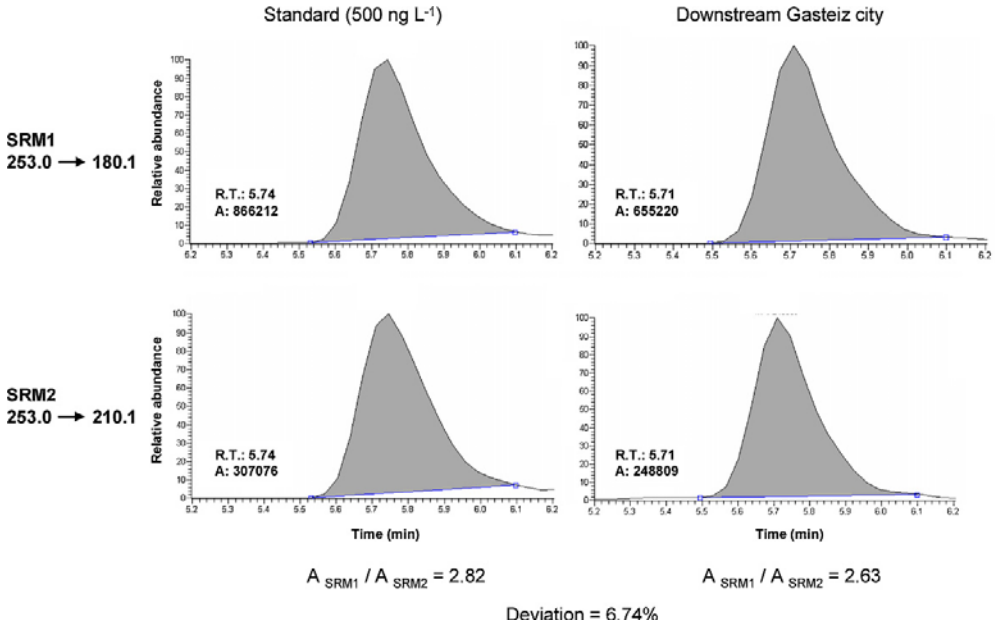

Fig. 3. Fulfilment of the identification criteria used for quantification of 10,11-epoxi carbamazepine in the location ZAD, situated downstream to the Gasteiz city.

4OH-diclofenac, mefenamic acid, the metabolite oxazepam glucuronide, chloramphenicol, hydrochlorothiazide, and the barbiturate pentobarbital, the range of linearity reached only until 500 ng L^{-1}. They were considered appropriate, since the concentrations of these microcontaminants in the validated matrixes (GW and RW) were expected to be within those ranges. The calibration curves obtained for both SRM1 and SRM2 were always linear with correlation coefficients (R^2) higher than 0.99 for all compounds.

3.4. Method limitations

The objective of developing an analytical method for simultaneous determination of pharmaceuticals and their metabolites/TPs in environmental waters (GW and RW) and wastewater (EWW and IWW) was successfully achieved just for the first two matrices (GW and RW). Up to our knowledge, until now TFC had been applied as a clean-up technique for biological samples like serum [26] and blood plasma [27–30], but never for environmental matrices like water.

As it was explained in Section 2.4, two mechanisms are taking place in the turbulent chromatography: a physical and a chemical one. The physical mechanism, based on diffusion phenomenon, is highly effective in case when matrix molecules are bigger than 8000 Da and analytes molecules smaller than 2000 Da weight. All the pharmaceuticals and TPs included in the study are far smaller than 2000 Da. In environmental waters main matrix component are soluble humic and fulvic acids. Although humic substances include hundreds of molecules, high portion are molecules with MW lower than 2000 Da. Consequently, those ones would not be flushed out through the first mechanism of clean-up. Proteins, carbohydrates, lipids and others biological macromolecules are generally bigger than 8000 Da, but unlikely to biological matrixes, they are not expected to occur at high concentrations in environmental and wastewaters. Nevertheless, smaller impurities can be removed by the chemical mechanism of clean-up, which affects the small molecules that diffused into the pores. But, since the spectrum of polarities and acidities of the target compounds is very wide, three different TFC columns were used in series to cover the properties of all of them. This resulted in a very efficient extraction, as it was demonstrated by the absolute recoveries in HPLC-grade water. But for the same reason, a large spectrum of molecules belonging to the matrix also may establish bonds with the functional groups, preventing an effective clean-up by the second mechanism.

Bearing all this in mind, a possible explanation for the observed unfeasibility of TFC to successfully clean-up complex wastewater samples, can be find in a scarce effectiveness during both mechanisms explained above. A similar behavior is expected for the first mechanism on other environmental samples, like sea water, extracts of sediments or sludge. The chemical mechanism is supposed to work better if the group of target compounds shares physico-chemical properties, since a more specific TFC column could be used and subsequently, the discrimination of matrix molecules would increase.

Other limitation observed was the elevated use of solvents, especially the loading mobile phase and the ones used for the cleaning routine in the injection port (syringe and injector). Therefore, this on-line technology cannot be considered fully “green”.

3.5. Monitoring results

The developed and validated method was applied to the analysis of river waters from the Ebro basin in the North of Spain, in order to demonstrate its applicability. Ten sampling sites were selected covering the whole Ebro River basin (5 at the Ebro River and 5 at the main tributaries). They were collected downstream to six major waste water treatment plants (WWTPs) like Tudela, Logroño, Pamplona, Zaragoza, Lleida and Tortosa, and other vulnerable sites, according to the proximity to big cities or industrial areas like Miranda de Ebro, Vitoria, Sabiñánigo and Monzón. Samples were collected during two weeks in the autumn 2010 (September–October). Concentrations found were summarized in Table 4. Out of the 77 compounds included in the method, 11 were

ubiquitous, among them the analgesics salycilic acid and propyphenazone, the psychiatric drug carbamazepine and its metabolites 2OH carbamazepine and 10,11-epoxi carbamazepine, the antihistaminic ranitidine, the antibiotics clarithromycin, sulfadiazine and trimothoprim, the cardiovascular drug propranolol and the antineoplastic tamoxifen. Levels detected were in general below 100 ng L^{-1}, except for 11 compounds (diclofenac, acetaminophen, phenylbutazone, gemfibrozil, the metabolite 10,11-epoxi carbamazepine, ranitidine and its metabolite ranitidine N-oxide, clarithromycin, atenolol, lisinopril and furosemide) which presented higher concentrations, specially in the tributaries.

The most relevant finding is the presence of carbamazepine metabolites and TPs 2OH carbamazepine, 3OH carbamazepine, 10,11-epoxi carbamazepine and acridin at concentrations surpassing those of the parent compound. Especially high levels are detected, in all analyzed samples, of the active metabolite 10,11-epoxi carbamazepine. Fig. 3 depicts the fulfilment of identification criteria for 10,11-epoxi carbamazepine in a sampling location downstream of Gasteiz city, where the highest concentration is determined (1670 ng L^{-1}).

Regarding the spatial distribution, for most of the compounds, the maximum and the mean concentration was shown to be higher among the tributaries than along the main river Ebro. That observation was also found in a previous work in the same river basin, published somewhere else [25]. This is attributed to the lower flow that generally characterized the tributaries, and consequently the lower dilution factors. As it was expected, highest levels of contamination by these compounds were found in sites after the areas more populated and/or industrialized, especially after the WWTPs of Vitoria, Pamplona and Zaragoza, all of them in tributaries. In general, levels found for every compound were in the same order of magnitude than in previous studies of occurrence in the Ebro River basin for similar compounds [25,31,32].

4. Conclusions

In this work, the successful application of the fully automated technique based on the turbulent chromatography technology, for the multi-residue analysis of 58 pharmaceuticals and 19 metabolites and TPs in environmental matrices like GW and RW is presented. However, the TFC technology was found to be unsuitable for clean-up and preconcentration of more complex samples such as raw and treated wastewater.

The use of small volumes (7.5 mL in total for ESI pos and ESI neg runs) facilitates sample storage when immediate analysis is not possible. Sample pretreatment includes only filtration, adjustment of pH and addition of surrogate standards. The use of isotopically labeled surrogates for most of the compounds was shown to be essential for an accurate quantification. Application of the method to the analysis of river samples from the Ebro basin showed a widespread occurrence of pharmaceuticals with levels generally lower than 100 ng L^{-1}.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2012.06.078.

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Publicación científica #4

"Multi-residue enantiomeric analysis of pharmaceuticals and their active metabolites in the Guadalquivir River basin (South Spain) by chiral liquid chromatography coupled with tandem mass spectrometry"

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Rebeca López-Serna, Barbara Kasprzyk-Hordern, Mira Petrović, Damià Barceló

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RESEARCH PAPER

Multi-residue enantiomeric analysis of pharmaceuticals and their active metabolites in the Guadalquivir River basin (South Spain) by chiral liquid chromatography coupled with tandem mass spectrometry

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Abstract This paper describes the development and application of a multi-residue chiral liquid chromatography coupled with tandem mass spectrometry method for simultaneous enantiomeric profiling of 18 chiral pharmaceuticals and their active metabolites (belonging to several therapeutic classes including analgesics, psychiatric drugs, antibiotics, cardiovascular drugs and β-agonists) in surface water and wastewater. To the authors' knowledge, this is the first time an enantiomeric method including such a high number of pharmaceuticals and their metabolites has been reported. Some of the pharmaceuticals have never been studied before in environmental matrices. Among them are timolol, betaxolol, carazolol and clenbuterol. A monitoring programme of the Guadalquivir River basin (South Spain), including 24 sampling sites and five wastewater treatment plants along the basin, revealed that enantiomeric composition of studied pharmaceuticals is dependent on compound and sampling site. Several compounds such as ibuprofen, atenolol, sotalol and metoprolol were frequently found as racemic mixtures. On the other hand, fluoxetine, propranolol and albuterol were found to be enriched with one enantiomer. Such an outcome might be of significant environmental relevance as two enantiomers of the same chiral compound might reveal different ecotoxicity. For example, propranolol was enriched with S(−)-enantiomer, which is known to be more toxic to *Pimephales promelas* than R(+)-propranolol. Fluoxetine was found to be enriched with S(+)-enantiomer, which is more toxic to *P. promelas* than R(−)-fluoxetine.

Keywords Chiral pharmaceutical · Metabolite · LC–MS/MS · River water · Wastewater · Enantiomer

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Introduction

It has been estimated that approximately 20–25 % of drugs used clinically are optically active and are distributed as racemates [1]. Enantiomers are compounds that cannot be superimposed. Thus, Ariens (1986) clearly and correctly classified racemic mixtures of drugs with a single chiral centre as mixtures of two drugs [1]. Chiral drugs can exist in the form of enantiomers, which have similar physicochemical

properties (lipophilicity, ionisation, molecular size) but differ in their biological properties and, therefore, in their potency, toxicity and biodegradation [2]. As a consequence of this stereoselectivity, the enantiomeric composition of chiral drugs can change significantly after their administration due to metabolism and excretion from human or animal body, subsequent biological wastewater treatment and/or biological degradation processes in the environment [2]. Furthermore, as a result of stereoselective transformation of chiral drugs, pharmacologically active and/or toxic transformation products can be formed, and they may be also chiral [2].

Thalidomide is a chiral drug that was widely prescribed in racemic form for morning sickness from 1957 to 1962. The S(−)-enantiomer was then found to be the cause of malformations in newborn babies. This tragic event resulted in a much greater emphasis on both the development of enantiomeric synthesis and separation processes. Finally, in 1992 and 1994, the FDA and EU respectively issued guidelines favouring the production of single enantiomer drugs rather than racemic form [3–7]. This has caused worldwide sales of chiral drugs in single-enantiomer form to continuously increase [2]. However, it has to be emphasised that racemates are still widely used. That is a cause for concern as enantiomers of a racemic drug can differ in toxicity. Furthermore, even a drug distributed in a single-enantiomer form can undergo chiral inversion to form an enantiomer of potentially higher toxicity. The establishment of multi-residue methods is crucial to obtain information regarding the cumulative presence of several groups of analytes at a particular place and time. This is of great importance as synergistic effects of different drugs on aquatic life might take place [2]. Despite the availability of methods for chiral analysis of drugs in biological matrices, it is difficult to directly utilise them in trace analysis of chiral drugs in the environment. This is because liquid chromatography coupled with ultraviolet detection (LC/UV) is usually utilised in the chiral analysis of drugs in biological matrices, and it is not sensitive and selective enough to be applied in environmental analysis, where usually tandem mass spectrometry has to be used. Direct transfer of chiral LC/UV method to liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method is also not always possible because the first one usually uses mobile phases which are incompatible with ESI-MS applications (non-volatile buffers, normal-phase solvents) [2]. Therefore, a few research groups have attempted to analyse chiral drugs in environmental samples by LC–MS/MS [8–13], but to date all have failed to provide a method capable of analysing a larger group of analytes. This paper presents the development of an analytical method for the simultaneous enantiomeric analysis of 15 chiral pharmaceuticals and their two main active chiral metabolites by LC–MS/MS, as well as its application in a monitoring programme of the Guadalquivir River basin (South Spain, 24 sampling locations) and five of the main wastewater treatment plants (WWTPs) located along the basin. To the authors' knowledge, this is one of the most comprehensive analytical enantiomeric methods applied to environmental matrices including the highest number of pharmaceuticals and their metabolites. In addition, this paper presents the first study of chiral drugs in environmental samples in Spain, providing information about the source and fate of these chiral residues at enantiomeric level in the Guadalquivir River basin.

Materials and methods

Selection of target analytes

Sixteen pharmaceuticals belonging to several therapeutic classes, including analgesics, psychiatric drugs, antibiotics, cardiovascular drugs and β-agonists, and two metabolites were chosen as target compounds. They are listed in Table S1 (Electronic Supplementary Material). The selection of target analytes was based on their occurrence and ubiquity in the aquatic environment, as well as their high human consumption in Spain. In the case of naproxen and timolol, only their S(+)- and S(−)-enantiomers, respectively, were included. European Pharmacopeia requires that these compounds are marketed in a single-enantiomer form. This is because R(+)-timolol is less effective than S(−)-timolol, and R(−)-naproxen presents hepatic toxicity. The selection of metabolites was based on their pharmacological activity and high urinary excretion by humans.

Chemicals

R/S(±)-ketoprofen, R/S(±)-ibuprofen and S(+)-naproxen (enantiomerically pure) were kindly supplied by Jescuder (Rubí, Spain). R/S(±)-fluoxetine (as hydrochloride), R/S(±)-ofloxacin, R/S(±)-flumequine, R/S(±)-atenolol, R/S(±)-sotalol, R/S(±)-metoprolol (as tartrate), R/S(±)-propranolol (as hydrochloride), S(−)-timolol (enantiomerically pure), R/S(±)-betaxolol, R/S(±)-carazolol, R/S(±)-pindolol, R/S(±)-albuterol and R/S(±)-clenbuterol (as hydrochloride) were purchased from Sigma-Aldrich (Steinheim, Germany). Metabolites R/S(±)-4OH propranolol (as hydrochloride) and R/S(±)-norfluoxetine were obtained from Toronto Research Chemicals Inc. (Ontario, Canada) and Cerilliant (Round Rock, TX, USA), respectively. All standards were of high purity grade (>90 %).

Isotopically labelled compounds (12) were used as surrogate standards. R/S(±)-fluoxetine-d5 was purchased from Sigma-Aldrich. R/S(±)-naproxen-d3, R/S(±)-ofloxacin-d8, R/S(±)-pindolol-d7, R/S(±)-timolol-d5 (as maleate salt),

R/S(±)-metoprolol-d7, R/S(±)-clenbuterol-d9, R/S(±)-propranolol-d7, R/S(±)-ketoprofen-13C-d3 and R/S(±)-flumequine-13C3 were obtained from Toronto Research Chemicals. R/S(±)-atenolol-d7 and R/S(±)-albuterol-d3 were obtained from CDN Isotopes (Quebec, Canada). R/S(±)-sotalol-d6 (as hydrochloride salt) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Both individual stock standard and isotopically labelled internal standard solutions were prepared on a weight basis in methanol (MeOH), except fluoroquinolones [R/S(±)-flumequine and R/S(±)-ofloxacin] which were dissolved in a H_2O/MeOH mixture (1:1) containing 0.2 % v/v hydrochloric acid, as they are only slightly soluble in pure MeOH. After preparation, standards were stored at −20 °C. Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of the substances were renewed every 3 months. A mixture of all pharmaceuticals was prepared by appropriate dilution of individual stock solutions in MeOH. Working standard solutions, prepared in mobile phase, were renewed before each analytical run. Working solutions were prepared in glass vials and standard mixtures in volumetric flasks, both wrapped with aluminium foil to prevent exposure to light. A separate mixture of isotopically labelled surrogates, used for internal standard calibration, was prepared in MeOH and further dilutions were made in mobile phase.

HPLC-grade MeOH, acetonitrile (ACN) and toluene, isopropanol (IPA, 99.8 %), formic acid (FA, 98 %) and ammonium acetate (NH_4Ac, 99 %) were supplied by Sigma Aldrich (Cambridge, UK). HPLC-grade H_2O was purchased from Fisher (Loughborough, UK). Hydrochloric acid (HCl, 37 %) was purchased from Merck (Darmstadt, Germany). All glassware was silanised with 5 % dimethylchlorosilane (DMCS) in toluene supplied by Sigma Aldrich (Cambridge, UK) to minimise sample loss through adsorption of basic analytes onto OH– sites present on glass surface.

Monitoring study site

Guadalquivir River basin is located in the South of the Iberian Peninsula (see Fig. 1). Its main river, Guadalquivir, flows 657 km in NE–SW direction into the Atlantic Ocean, in a large estuary. It drains a total area of 57,527 km^2. Approximately 4,107,598 people live in the river basin area. As a consequence of such a high population, the river receives many inputs from both natural and anthropogenic origin that may cause deterioration of water quality. Its natural environment is one of the most varied in Europe, containing representatives of half of the continent's plant species and nearly all those of the North African region. The estuary has an added ecological value due to the presence of the Doñana National Park, an important and protected wetland area, which has undergone anthropogenic transformations and suffers from metal inputs. The area is under the influence of Mediterranean weather, i.e. warm-hot temperatures (annual average 16.8 °C) and irregular rainfall regime (annual average 550 L/m^2) characterised by long-term droughts interspersed by torrential rain events. The river is navigable up as far as Seville (about 90 km upstream), a major inland port. But this navigation also leads to a serious environmental problem due to erosion and pollution. The lower Guadalquivir River basin is also impacted by reservoirs and dams, and its regime is rather artificial. Additionally, more than 700,000 ha of its basin are devoted to agriculture, with very high production of rice, especially in the river's lower course that traverses extensive marshlands, olives or fruits, with corresponding environmental effects on the river.

Twenty four sampling locations were selected along the basin (Table S2, Electronic Supplementary Material) to depict the contamination gradient at the end of October and beginning of November 2011 when the flow in the basin was medium-low. Fifteen sites belonged to tributaries and nine to the main Guadalquivir River. The sites covered all the strategic points along the basin, including sites near the river sources, like BOR, MAG, BEM, CAC, GUA2 and GUA3; others are situated downstream of WWTPs of important urban areas like Córdoba (GUA4), Sevilla (GUA6), Loja (GEN1) and Morón de la Frontera (GUAA). GUA1, GUAL, PIC and HER are in important agricultural olive, orange or cereal areas. Some other sites were selected in the estuary at the mouth of the main Guadalquivir River (GUA7, GUA8 and GUA9). Samples were also collected from five wastewater treatment plants located in the Guadalquivir River basin. All WWTPs utilise an activated sludge technology.

Sample pre-treatment

Sample collection 24-h composite influent wastewater (IWW), 24-h composite effluent wastewater (EWW) and grab river water samples were collected in amber glass bottles and transported to the laboratory in the dark in chambers cooled with ice. Wastewater samples were subsequently vacuum-filtered through GF/D 2.7 μm and GF/F 0.7 μm filters (Whatman, Fairfield, CT, USA) and were stored in the dark at −20 °C until pre-concentration. River water samples were filtered only through GF/F 0.7 μm filters.

Solid phase extraction (SPE) SPE was undertaken according to methodology published by Bagnall et al. [13]. Briefly, for the pre-concentration of water samples, a Baker vacuum system (J.T. Baker, The Netherlands) was used. Oasis HLB cartridges (60 mg, 3 mL) from Waters Corporation (Milford, MA, USA) were conditioned with 2 mL of MeOH and equilibrated with 2 mL of water at pH 7.5 (adjusted if necessary with NaOH). A volume of 500 mL of river water or 100 mL of sewage water, both at pH 7.5 (adjusted if necessary with

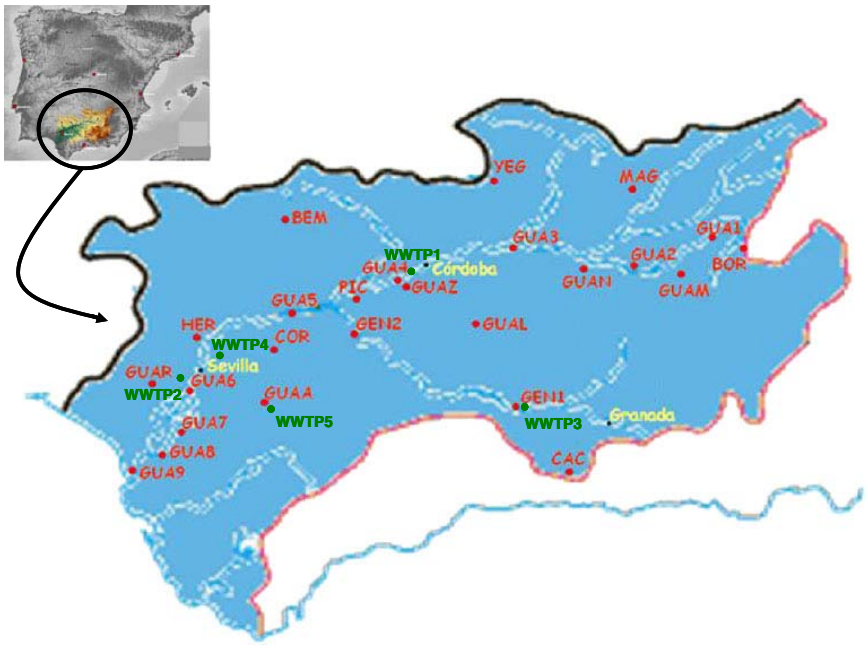

Fig. 1 Sampling map

NaOH) and spiked with 50 ng of each surrogate standard, were passed through the cartridge at a flow rate of approximately 5 mL min^{-1}. After sample pre-concentration, cartridges were dried under vacuum for 15–20 min to remove excess of water, and then were frozen and kept at −20 °C until elution (<2 months). Analytes were eluted with 4 mL of MeOH. Extracts were then evaporated to dryness under a gentle nitrogen stream (<5 psi) at 40 °C with a TurboVap evaporator (Caliper, Preston Brook, UK) and reconstituted in 0.5 mL of mobile phase. All samples were filtered through 0.2 μm PTFE Puradisc 13-mm filters (Whatman, Kent, UK) and transferred to polypropylene 0.3-mL-capacity vials (Waters, Manchester, UK).

LC–MS/MS analysis

Separation of enantiomers of chiral drugs was undertaken with an Acquity UPLC system (Waters) equipped with a Chirobiotic V 250×2.1 mm, I.D. 5 μm column and Chirobiotic V 20×1.0 mm, I.D. 5 μm guard column (Sigma Aldrich, Cambridge, UK). The optimized mobile phase consisted of 4 mM NH_4Ac, 0.005 % FA and 99.995 % MeOH working in isocratic mode at a flow rate of 0.1 mL min^{-1}. The total time of chromatographic analysis was 66 min and the injection volume was 20 μL. The column was kept at room temperature (25 °C) and samples were preserved in the autosampler at 4 °C.

Identification and quantification of chiral drugs was undertaken with an Acquity Xevo TQD, a triple quadrupole MS equipped with an ESCi source (Waters). For most of the compounds, two selected reaction monitoring (SRM) transitions between the precursor ion and the two most abundant fragment ions were monitored (full list of SRMs and instrumental conditions is given in Table S1, Electronic Supplementary Material). This enables compliance with the requirements set by the European Union (EU) regulations (EU Commission Decision 2002/657/EC) [14] related to identification and confirmation of contaminants analysed by LC–MS/MS. However, in the case of R/S(±)-norfluoxetine, due to its poor fragmentation, only one transition was monitored. One transition was also monitored for the isotopically labelled standards. Settings for source-dependent parameters were as follows: capillary voltage, 3.5 kV; desolvation temperature, 350 °C; desolvation gas flow, 650 L h^{-1}; and cone gas (N_2) flow, 0 L h^{-1}. To achieve the optimum mass sensitivity/selectivity ratio, low mass and high mass resolution were set at 7.9 and 14.3, at the first quadrupole, and 12.0 and 14.7, at the third quadrupole, respectively. Nitrogen was used as the desolvating/nebulising gas provided by a high-purity nitrogen generator from Peak Scientific Instruments Ltd. (Renfrewshire, UK). Argon (99.999 %) was used as the collision gas. MassLynx 4.1 software from Waters was used to control the Acquity UPLC and the Acquity Xevo TQD, as well as to process the data.

Method validation

In order to avoid false-positive measurements, all validation parameters—accuracy, matrix effects, sensitivity, precision,

enantiomeric fraction (EF) and chromatographic resolution (R_s)—were calculated for isotopically labelled analogues of studied analytes. Linearity range was calculated for the non-labelled analytes. Twelve isotopically labelled surrogates (see Table S1, Electronic Supplementary Material) were studied in several matrices: surface water (SW), wastewater effluent (EWW) and wastewater influent (IWW). Isotopically labelled compounds were not available for ibuprofen, betaxolol, carazolol, norfluoxetine and 4OH propranolol. Therefore, results obtained for these compounds should be treated on a semi-quantitative basis.

Method recovery and precision Absolute recoveries were determined for all studied environmental matrices by spiking samples at environmentally relevant concentrations of 100 ng L^{-1} for SW (n=24) and 500 ng L^{-1} for both EWW and IWW (n=5). Recoveries were determined as percentages by comparing the peak areas of analytes obtained with the optimized method for spiked environmental samples with the peak areas of analytes obtained from direct injection of equivalent amounts of standards in MeOH.

Sensitivity The limits of detection (LODs) and quantification (LOQs) of the developed method were experimentally measured with the isotopically labelled surrogates as the concentration of analyte giving a signal-to-noise ratio of 3 and 10, respectively.

Enantiomeric fraction EF is the relative concentration of each enantiomer in a chiral compound and can be calculated using the following equation:

$$\mathrm{EF} = \frac{\mathrm{E}1}{\mathrm{E}1 + \mathrm{E}2} \quad (1)$$

where E1 and E2 represent peak areas of the (+) and (−) enantiomers, respectively, or the first- or the second-eluted enantiomers under defined chromatographic conditions if the elution order is not known. Ideally, EF equals 1 or 0 in the case of a single enantiomer form and 0.5 in the case of racemate. For further discussion on EF calculation, please go to 'Method validation' section.

Chromatographic resolution R_s was calculated using the following equation:

$$R_s = \frac{2(t_{r2} - t_{r1})}{w_1 + w_2} \quad (2)$$

where t_{r1} and t_{r2} are the retention times of the first and second eluted enantiomers, and w_1 and w_2 are the widths of their signals (peaks) at the base line.

Results and discussion

Method optimisation

Optimisation of chiral liquid chromatography

In order to obtain the best chromatographic resolution of enantiomers while maintaining satisfactory sensitivity of the method and acceptable analysis time, several mobile phase conditions were studied. Chromatographic separation was tested in polar organic (organic solvent—MeOH or ACN), polar ionic mode (organic solvent—MeOH or ACN modified with varying concentrations of 4–30 mM NH_4Ac, 0.005–0.1 % FA and 0–5 % H_2O) and reversed phase mode (organic solvent—MeOH modified with varying concentrations of 4–30 mM NH_4Ac, 0.005–1.48 % FA and 5–80 % H_2O). A wide range of pH of mobile phase (3.5–8.0) was also studied as pH can affect ionisation of both chiral selectors and ionic/ionisable analytes.

It was observed that the mobile phase consisting of organic solvents only (MeOH and ACN) did not allow for resolution of enantiomers. Additives such as NH_4Ac, H_2O and FA were needed for required enantioselectivity and retention times. Little difference was observed between MeOH and ACN as solvents. As a result, the mobile phase consisting of 4 mM NH_4Ac, 0.005 % FA in MeOH (polar ionic mode) was found to provide the best enantioselectivity, acceptable retention times and sensitivity. It provided enantiomeric separation for 12 pharmaceuticals: ibuprofen, fluoxetine, salbutamol, clenbuterol, betaxolol, pindolol, propranolol, atenolol, metoprolol, sotalol, carazolol, timolol and 4OH propranolol. Partial enantiomeric separation was achieved for norfluoxetine. Enantiomers of ketoprofen, naproxen and fluoroquinolone flumequine remained unresolved, although they eluted within the method run time. Ofloxacin did not elute as a well-defined peak during the optimised chromatographic run and therefore was finally excluded from the method.

In summary, the comprehensive method development revealed that the Chirobiotic V stationary phase allowed only for the separation of basic compounds (with pK_a >8). In contrast, enantiomers of acidic analytes (with pK_a <5) were not resolved and eluted with very short retention times indicating weak interactions with the stationary phase.

Optimisation of mass spectrometry detection

A pair of enantiomers, due to their identical physicochemical properties, is indistinguishable in mass spectrometry as separation of chemicals takes place according to their m/z ratio. Therefore, optimised mass spectrometry parameters (e.g. SRM transitions) were in this method identical for each

pair of enantiomers. Selection of parent ions and optimum ionisation mode for each compound were performed by infusing 100 μg L^{-1} individual standard solutions in MeOH in a full-scan mode. All the 18 compounds investigated along with 13 isotopically labelled surrogates showed a higher response in ESI pos mode than in ESI neg mode, as expected. In all cases, $[M+H]^+$ were selected as parent ions. The compound-dependent cone voltage was optimised in full-scan mode by the automatic tuning provided by the MassLynx software (IntelliStart). Identification of the two most abundant fragment ions and the selection of the optimum collision energy for each fragment in the product ion scan mode was also undertaken with IntelliStart. For quantitative analysis, two SRM transitions between the precursor ion and the two most abundant fragment ions were established for all compounds with the exception of norfluoxetine, for which only one SRM transition could be recorded, due to its poor fragmentation. The most intensive transition was used for quantitation purposes, whereas the second transition was used to confirm the presence of target compounds in the samples. Thus, the number of identification points required to confirm the detection of target analytes, according to the EU regulations (EU Commission Decision 2002/657/EC), was achieved (four IP, one for precursor ion and 1.5 for each transition product). For isotopically labelled standards, only one transition was monitored. Besides the monitoring of the SRM transitions, other identification criteria were used for quantification:

(a) The difference in the retention time between every enantiomer (or racemic compound if not resolved) and its corresponding internal standard must be kept within ±2 % in samples with respect to the difference in the calibration curve solutions.
(b) The relative abundances of the two selected SRM transitions monitored for each enantiomer (or racemic compound if not resolved) in the sample must be within ±20 % of that ratio in the analytical standards.

Both parameters (retention time and ratio SRM1/SRM2) are given in Table S1 (Electronic Supplementary Material). Dwell time was set at 0.076 for all analytes. A temperature of 350 °C was chosen as the suitable desolvation temperature. Representative SRM chromatograms of a standard mixture of analytes at a concentration of 100 μg L^{-1} obtained using the optimum method conditions are illustrated in Fig. 2.

Method validation

Method validation data are presented in Table 1 and 2.

Method recovery and matrix effects As can be observed in Table 1, absolute recoveries in SW, EWW and IWW samples were >80 % in the case of most of the analytes, which confirms a very good performance of the method. Absolute recoveries show the overall efficiency of the whole analytical procedure. Matrix effects, along with the SPE recovery, are one of the most important parameters contributing to the overall performance of the method as well as a possible source of significant errors if not accounted for during both qualitative and quantitative measurements. Phenomena occurring during the SPE process are expected to affect a pair of enantiomers equally regardless of the matrix involved. In contrast, matrix effects (both suppression and less common signal enhancement) could affect a pair of enantiomers to a different extent due to their physical separation in time. In this work, stereoselective signal suppression was observed and was found to be more significant in a more complex matrix such as IWW thus affecting the following compounds: metoprolol, clenbuterol, sotalol and timolol, and was particularly strong in the case of pindolol and propranolol (Table 1). For example, in the case of propranolol-d7 absolute recovery denoted 102 % and 100 % in SW for S(−)- and R(+)-enantiomer, respectively, indicating a lack of stereoselectivity. However, in IWW absolute recovery denoted 44 % and 94 % in the case of S(−)- and R(+)-enantiomer, respectively, indicating stereoselectivity resulting in much higher suppression of the signal of S(−)-enantiomer.

Any errors, losses or matrix effects occurring during the overall analytical process (and possibly affecting a pair of enantiomers to a different extent) were compensated for with the usage of the isotopically labelled surrogates, which were added to the samples at the beginning of the analytical protocol (before the SPE). In the case of five analytes with no isotopically labelled surrogate (ibuprofen, betaxolol, carazolol, norfluoxetine and 4OH propranolol), the most appropriate isotopically labelled standard was selected from those available. The choice was based on similarity in structure and proximity in retention time (Table S1, Electronic Supplementary Material).

The above discussion clearly indicates the critical importance of the usage of labelled analogues of analytes as internal standards in order to compensate for any stereoselective phenomena occurring. This is especially important if an enantiomeric determination is undertaken with the usage of mass spectrometry equipped with the ESI interface, which is well known to be highly susceptible to matrix effects.

Sensitivity Table 1 shows the LODs and LOQs calculated for mobile phase, SW, EWW and IWW. The developed method was found to be very sensitive towards target analytes in all matrices studied. Instrumental limits of quantification were on average <10 pg. However, as expected, a slight decrease in sensitivity was observed with an increase in the complexity of the matrix due to the signal suppression and resulting higher background noise. LOD and LOQ levels were in general in the low nanogram per litre levels (<10 ng L^{-1}).

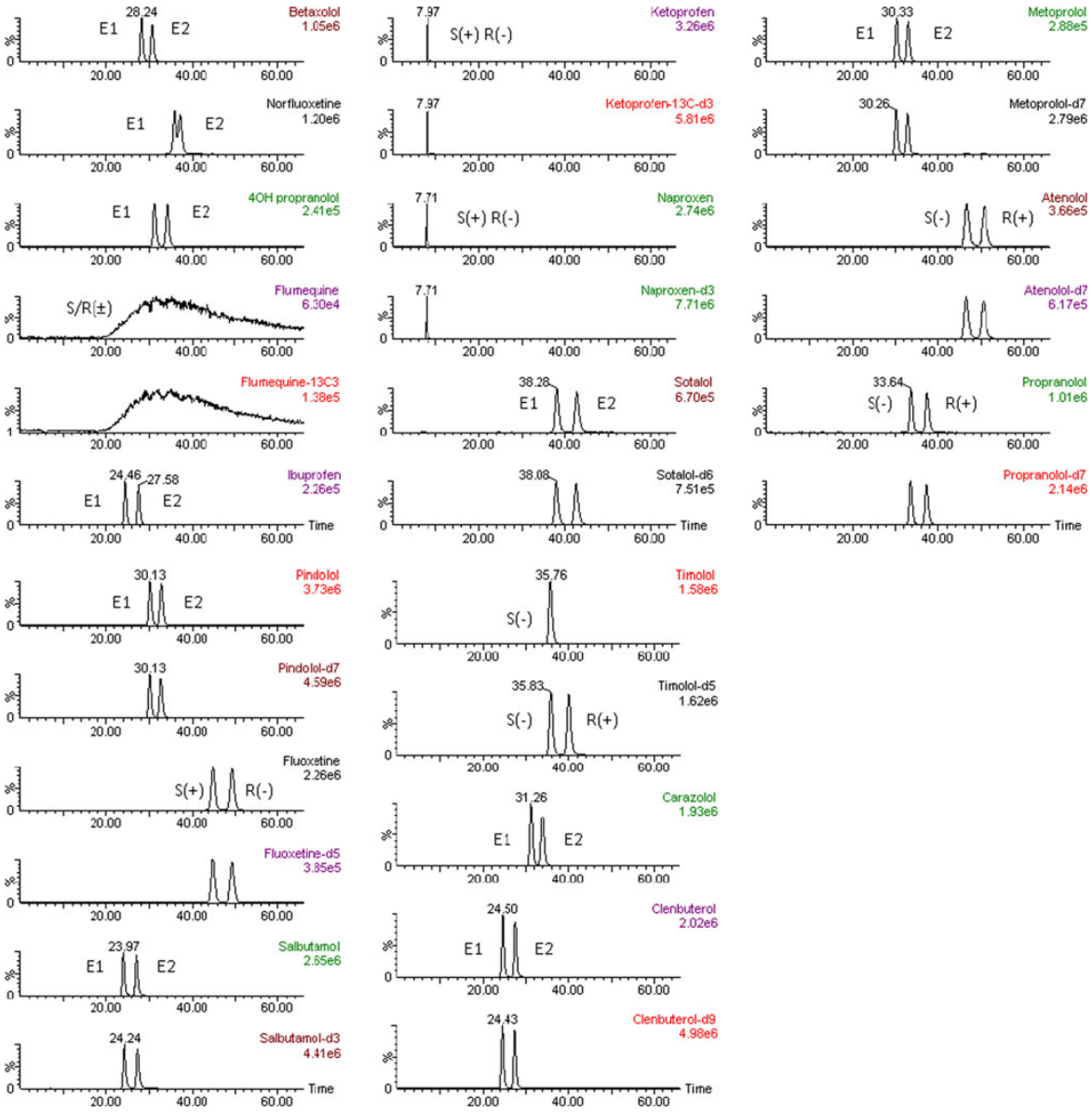

Fig. 2 LC–MS/MS chromatograms for studied chiral pharmaceuticals and their metabolites (concentration, 100 μg L^{-1})

Precision The overall method repeatability, calculated as the relative standard deviation (RSD) of the replicate (n=12) analysis of SW water spiked with a mixture of the isotopically labelled surrogates (100 ng L^{-1}), was satisfactory with %RSD <20 % for most of the compounds in the case of both intra- and interday measurements (Table 2).

Linearity Quantification based on peak areas was performed by the internal standard (IS) approach. For each analyte, the corresponding or the most appropriate isotopically labelled surrogate was used as internal standard. A ten-point calibration curve was constructed, using least-squares linear regression analysis, from mixtures of target pharmaceuticals in mobile phase at concentrations ranging from 0.01 μg L^{-1} (or the limit of quantification if higher) to 250 μg L^{-1}. The calibration curves obtained for both SRM1 and SRM2 were always linear with correlation coefficients (R^2) exceeding on average 0.998 for target compounds (Table 2).

Table 1 Validation parameters for chiral pharmaceuticals and their metabolites: absolute recovery, limits of detection and quantification

Analyte	Absolute recovery (%)			LOD (ng L^{-1})				LOQ (ng L^{-1})			
	SW	EWW	IWW	Instrumental (pg)	SW	EWW	IWW	Instrumental (pg)	SW	EWW	IWW
rac-Flumequine-13C3	97.5	98.4	101.0	132.9	3.47	10.62	10.61	232.56	9.27	28.31	28.28
Albuterol-d3 E1	55.9	94.8	115.8	3.92	0.23	0.12	0.21	8.55	0.61	0.31	0.57
Albuterol-d3 E2	69.4	99.2	108.5	4.69	0.16	0.10	0.08	10.31	0.44	0.28	0.22
rac-Ketoprofen-13C-d3	97.8	92.3	27.3	1.21	0.23	0.91	1.24	1.83	0.60	2.42	3.30
Pindolol-d7 E1	95.5	93.3	72.3	2.75	0.09	0.09	0.18	10.57	0.25	0.23	0.48
Pindolol-d7 E2	99.3	76.8	50.5	2.87	0.13	0.09	0.53	11.02	0.34	0.23	1.41
(S−)-Propranolol-d7	102.1	75.4	43.8	2.29	0.39	0.36	0.46	1.13	1.04	0.95	1.22
(R+)-Propranolol-d7	99.7	92.0	93.8	2.57	0.29	0.22	0.40	1.31	0.77	0.59	1.06
(S−)-Atenolol-d7	99.7	105.0	97.2	8.95	0.45	0.48	1.23	23.20	1.20	1.28	3.28
(R+)-Atenolol-d7	102.1	103.6	88.1	9.77	0.48	0.48	1.27	25.43	1.28	1.28	3.38
Metoprolol-d7 E1	97.4	96.8	81.8	3.39	0.14	0.34	0.11	11.79	0.37	0.91	0.30
Metoprolol-d7 E2	99.9	96.2	62.5	3.60	0.22	0.36	0.38	12.48	0.58	0.96	1.02
Clenbuterol-d9 E1	100.9	107.3	70.6	1.00	0.10	2.91	10.03	2.46	0.27	7.75	26.74
Clenbuterol-d9 E2	99.8	97.0	80.4	1.07	0.09	2.49	2.33	2.62	0.23	6.64	6.22
Sotalol-d6 E1	100.9	101.0	101.5	4.45	0.62	1.02	0.82	1.75	1.65	2.72	2.18
Sotalol-d6 E2	99.3	113.1	97.2	4.73	0.49	0.75	0.55	1.81	1.31	1.99	1.47
(S−)-Timolol-d5	99.8	102.5	69.2	1.40	0.27	0.27	0.38	0.76	0.71	0.72	1.00
(R+)-Timolol-d5	105.9	99.6	98.4	1.53	0.16	0.15	0.18	0.79	0.44	0.41	0.49
rac-Naproxen-d3	99.2	63.9	13.1	7.82	0.36	1.49	2.27	36.45	0.96	3.98	6.06
(S+)-Fluoxetine-d5	100.1	101.9	105.7	6.16	0.43	0.47	1.11	7.42	1.14	1.25	2.95
(R−)-Fluoxetine-d5	100.7	103.6	108.0	6.54	0.44	0.54	1.04	7.52	1.18	1.45	2.78

Enantiomeric fraction The EFs calculated (with Eq. 1) for the mixture of racemic isotopically labelled surrogates in mobile phase, as well as in SW, EWW and IWW are shown in Table 2. EFs of 0.5 were expected in all studied matrices. However, it was interesting to observe how an increase in the complexity of the matrix resulted in EF values deviating from the 0.5 for certain compounds: albuterol, pindolol, clenbuterol and especially propranolol, metoprolol and timolol. This phenomenon can be attributed to the stereoselective matrix effects occurring. For example, EF for propranolol-d7 was 0.5 in the standard solution, 0.58 in surface water and 0.66 in IWW, which is the most complex matrix.

As mentioned before, this phenomenon can and should be compensated by using labelled analogues of standards, e.g. deuterated internal standards. Thus, to avoid the above-mentioned problems and to limit differential instrument response for each enantiomer, the modified Eq. 1 is recommended for EF calculation:

$$EF_{rel} = \frac{E1_{rel}}{E1_{rel} + E2_{rel}} \text{ and } E1_{rel} = \frac{E1}{E1_{IS}}, E2_{rel} = \frac{E2}{E2_{IS}} \quad (3)$$

where $E1_{IS}$, $E2_{IS}$ represent corresponding peak areas of internal standards (important note—internal standards should be isotopically labelled, e.g. deuterated or C13 analogues of analytes).

The modified Eq. 2 was utilised in environmental monitoring, which is discussed below. This is to avoid any errors resulting from stereoselective signal suppression.

Chromatographic resolution R_s calculated for the mixture of isotopically labelled surrogates in mobile phase as well as in SW, EWW and IWW are shown in Table 2. The R_s values are compound dependent and denoted on average 1.0, which indicates 2 % overlap and is generally accepted for quantitative analysis. Norfluoxetine was an exception, with R_s of 0.38, and therefore, its determination should be considered on a semi-quantitative basis.

Monitoring results

The results of the monitoring campaign including 24 river sampling sites and five WWTPs are shown in Table S3 (Electronic Supplementary Material).

Environmental concentrations of chiral pharmaceuticals and their metabolites

Out of 18 target compounds monitored (see Table S1), three analgesics (ketoprofen, ibuprofen and naproxen) were found to be ubiquitous and were detected in all analysed samples.

Table 2 Validation parameters for chiral pharmaceuticals and their metabolites: repeatability and reproducibility, linearity and range, enantiomeric fractions and chromatographic resolution

Analyte	Repeatability intraday (%RSD) SW	Repeatability interday (%RSD) SW	Linearity			EF				R_s			
			R	R^2	Range (ng L^{-1})	Calibration curve	SW	EWW	IWW	Calibration curve	SW	EWW	IWW
rac-Flumequine-13C3	2.2	25.3	0.9990	0.9979	LOQ–250								
Albuterol-d3 E1	22.1	2.7	0.9993	0.9986	LOQ–250	0.49	0.48	0.49	0.44	1.05	0.97	1.00	0.94
Albuterol-d3 E2	19.0	41.2	0.9998	0.9996	LOQ–250								
rac-Ketoprofen-13C-d3	1.1	0.6	0.9992	0.9983	LOQ–250								
Pindolol-d7 E1	19.7	23.0	0.9983	0.9967	LOQ–250	0.50	0.55	0.56	0.58	0.91	0.61	0.74	0.66
Pindolol-d7 E2	7.5	1.7	0.9990	0.9980	LOQ–250								
(S−)-Propranolol-d7	12.7	19.1	0.9994	0.9989	LOQ–250								
(R+)-Propranolol-d7	8.5	52.6	0.9998	0.9996	LOQ–250	0.50	0.58	0.62	0.66	1.00	1.02	0.99	1.02
(S−)-Atenolol-d7	9.5	3.9	0.9990	0.9980	LOQ–250								
(R+)-Atenolol-d7 E2	14.8	11.2	0.9990	0.9979	LOQ–250	0.50	0.51	0.51	0.51	0.97	0.86	0.86	0.88
Metoprolol-d7 E1	18.9	5.5	0.9989	0.9978	LOQ–250	0.50	0.56	0.57	0.60	0.91	0.63	0.70	0.60
Metoprolol-d7 E2	10.2	7.3	0.9996	0.9992	LOQ–250								
Clenbuterol-d9 E1	6.8	2.3	0.9992	0.9983	LOQ–250	0.50	0.48	0.49	0.43	1.11	0.99	1.01	0.98
Clenbuterol-d9 E2	6.6	51.8	0.9996	0.9992	LOQ–250								
Sotalol-d6 E1	5.1	40.1	0.9995	0.9991	LOQ–250	0.49	0.46	0.45	0.49	1.08	0.89	0.93	0.91
Sotalol-d6 E2	13.8	22.8	0.9994	0.9988	LOQ–250								
(S−)-Timolol-d5	22.4	54.3	0.9994	0.9989	LOQ–250								
(R+)-Timolol-d5	4.3	21.8				0.51	0.57	0.60	0.58	1.13	0.91	0.92	0.89
rac-Naproxen-d3	0.7	1.5	0.9932	0.9864	LOQ–250								
(S+)-Fluoxetine-d5	20.3	3.9	0.9990	0.9980	LOQ–250	0.50	0.49	0.50	0.53	1.08	0.90	0.85	0.84
(R−)-Fluoxetine-d5	23.8	15.2	0.9989	0.9978	LOQ–250								

Another 11 compounds, including metabolite norfluoxetine, were detected in at least one sample. Thus, only four substances were not detected at any of the sampling locations. Three of them were parent compounds, i.e. betaxolol, carazolol and clenbuterol, and one metabolite, 4OH propranolol.

Average concentrations of pharmaceuticals were generally far below 100 ng L^{-1} (Fig. 3). However, there were a few exceptions: anti-inflammatory naproxen, ketoprofen and ibuprofen, and β-blocker atenolol, which were recorded at average concentrations exceeding 1 μg L^{-1}. Ibuprofen was quantified at average concentrations of 622, 1,942 and 6,559 ng L^{-1} in SW, EWW and IWW, respectively. Naproxen was quantified at 129, 626 and 4,073 ng L^{-1} in SW, EWW and IWW, respectively. The analgesic ketoprofen and the β-blocker atenolol were quantified in sewage at 281 and 410 ng L^{-1} (ketoprofen) and at 586 and 1,288 ng L^{-1} (atenolol) in EWW and IWW, respectively. Thus, average concentrations observed here for ibuprofen and naproxen were significantly higher than the ones observed in other Mediterranean rivers, like the Ebro River [15–17]. This might be because the sampling campaign was carried out when the stream flows in the whole basin were at medium-low levels. In contrast, average concentrations in sewage waters presented here were in general similar to other WWTPs from other populated parts of Spain like Catalonia, which was previously monitored by Gross et al. [15].

The metabolites included in this work were present in studied samples at the low nanograms per litre level, with maximum concentration denoting 18 ng L^{-1} in IWW in the case of norfluoxetine. It is worth mentioning that norfluoxetine was found at similar levels as fluoxetine in all matrices except in EWW, where fluoxetine was mostly more concentrated with an average of 11 ng L^{-1} when compared with <1 ng L^{-1} of norfluoxetine. 4OH propranolol was not quantified in any of the analysed environmental samples despite the fact that propranolol was found at levels reaching tens of nanograms per litre in sewage. Previous studies by Huerta-Fontela et al. [18] reported levels below the limit of quantification for fluoxetine as well as norfluoxetine in Llobregat River, another Spanish Mediterranean river. Another study by the same authors [19] also showed the absence of both fluoxetine and norfluoxetine in sewage waters from WWTPs in the North-East of Spain. To the authors' knowledge, there is no published data of concentrations of 4OH propranolol either in SW or wastewater.

Enantiomeric profiling of chiral pharmaceuticals and their metabolites in the environment

Metabolism of chiral NSAIDs and β-blockers in humans is known to be stereoselective [2]. However, in this investigation, ibuprofen, atenolol, sotalol and metoprolol were present generally as racemic mixtures in studied environmental samples (Fig. 4). Ibuprofen was present as a racemate in most of the samples, except for the SW *GEN2*, and the IWWs of WWTP1 and WWTP4. In those three cases, the second eluting enantiomer was present at higher concentrations. A similar situation was observed in the case of atenolol. In several surface water samples from the main Guadalquivir River (i.e. GUA3, GUA8 and GUA9), a significant enrichment of atenolol with S(−)-enantiomer was observed. This is in agreement with other studies [8, 12, 13, 20]. Furthermore, stereoselective transformation of atenolol was observed in WWTP1 and 4 (utilising activated sludge treatment in both cases) leading to an enrichment of atenolol with S(−)-enantiomer. This outcome concurs with work reported by Nikolai et al. [8] and MacLeod et al. [9] where an enrichment of atenolol with S(−)-enantiomer took place. In contrast, an enrichment of atenolol with R(+)-enantiomer was observed by Kasprzyk-Hordern et al. [12] during wastewater treatment. This was especially evident during activated sludge treatment. No or low stereoselectivity was observed in the case of trickling filters [12]. Other β-blockers such as sotalol and metoprolol were also the most commonly present in surface water and wastewater as racemic mixtures (EF_{rel}=0.5). Similar results were obtained for metoprolol by Bagnall et al. [13] and Nikolai et al. [8]. It is, however, important to emphasise that absolute EFs (which do not account for matrix effects) calculated for these analytes were, in the present study, frequently >0.55 for metoprolol and <0.45 for sotalol indicating, erroneously, non-racemic composition, which in actual fact resulted from matrix effects and not, as one could suggest, non-racemic environmental composition. The above discussion confirms the importance of the usage of isotopically labelled internal/surrogate standards in chiral analysis of complex environmental samples.

In contrast, the enantiomeric profiles of propranolol, fluoxetine and albuterol showed a predominance of one enantiomer. Causes for a non-racemic composition can vary depending, among other factors, on the type of matrix. In the IWWs, the imbalance can be due a different rate of excretion of both enantiomers in the target organism (e.g. humans). Change in enantiomeric composition observed in EWW when compared to IWW might result from stereoselective microbial processes occurring during biological treatment (e.g. activated sludge). Stereoselective biotransformation can also occur in the environment. Thus, propranolol, although affected by matrix effects, revealed, as expected due to stereoselective metabolism in humans [2], non-racemic composition with an excess of S(−)-enantiomer in most of the EWW, IWW and SW samples. This could be a significant finding as S(−)-propranolol has been found to be more toxic to *Pimephales promelas*, which is thought to be due to suitable receptors, which are shared by many other organisms including humans [21]. Similar results were also

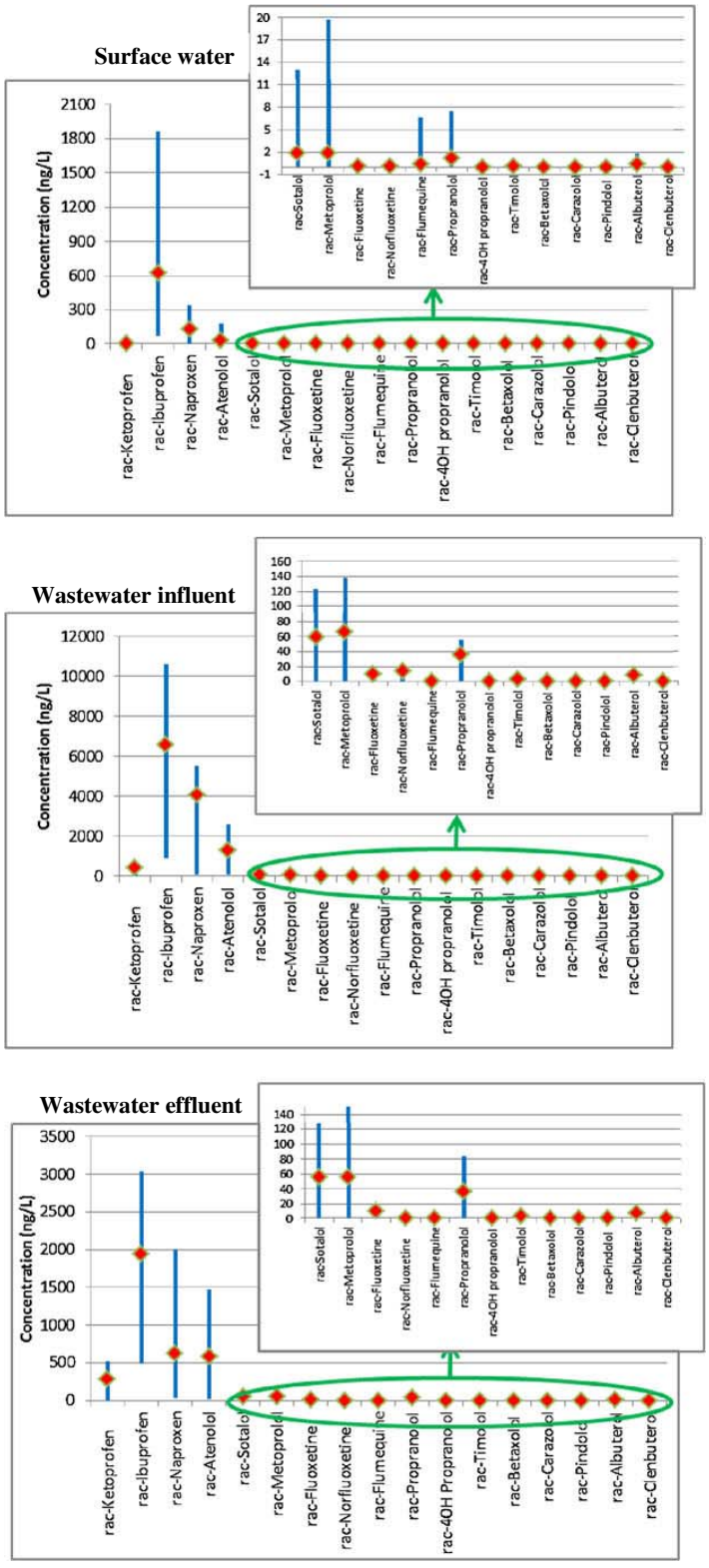

Fig. 3 Concentrations of chiral pharmaceuticals and their metabolites in surface water and wastewater

Fig. 4 Enantiomeric profile of chiral pharmaceuticals and their metabolites in the Guadalquivir River basin

recorded for propranolol in sewage waters monitored by Nikolai et al. [8], MacLeod at al. [9] and EWW and SW by Bagnall et al. [13]. In contrast, Fono and Sedlak [22] reported propranolol to be racemic in the influent of studied WWTPs, but enrichment in S(−)-enantiomer took place as a result of activated sludge treatment. However, in this work, no stereoselectivity was observed in the transformation of propranolol during wastewater treatment in any of the WWTPs studied in this work. In the case of fluoxetine (Fig. 4), an excess of S(+)-enantiomer was recorded in both wastewater and surface water samples. This is probably because of stereoselective metabolism of fluoxetine in humans [2]. An excess of S(+)-fluoxetine was also observed in sewage monitored by Barclay et al. [23], but the opposite situation [an excess of R(+)-enantiomer] was recorded by MacLeod et al. [9]. Enrichment of fluoxetine with *S*(+)-enantiomer is of potentially significant ecotoxicological consequence as toxic effects of fluoxetine enantiomers are species dependent with *S*-fluoxetine being more toxic than *R*-fluoxetine in *P. promelas* [24]. Furthermore, in this work, norfluoxetine, a main chiral and active metabolite of fluoxetine, was also analysed. It was found to be non-racemic with enantiomeric fractions varying significantly in different samples. This was probably due to the poor enantiomeric resolution of norfluoxetine and its very low environmental concentrations. Similarly, Barclay et al. [23] also observed enrichment of norfluoxetine with one enantiomer in wastewater samples studied. Albuterol was commonly found in the environment enriched with the second-eluting enantiomer (Fig. 4). Enrichment of albuterol with E2-enantiomer was also observed by MacLeod et al. [9] who used the same chromatographic column. Enantiomeric profiling of 4OH propranolol, betaxolol, carazolol, pindolol, timolol and clenbuterol could not be undertaken as these pharmaceuticals were either not identified or quantified in studied environmental matrices.

Spatial distribution

Figure 5 shows the cumulative concentrations of all pharmaceuticals monitored in the Guadalquivir River basin. The highest concentrations of both parent compounds and their metabolites were found in untreated sewage water samples. Nonetheless, concentrations for WWTP3 were clearly lower than the ones in other WWTPs. In fact, levels in IWW3 and EWW3 were comparable to the ones found in SW, with cumulative concentrations not reaching 2,000 ng L^{-1}. This is because WWTP3 serves a small population of <22,000 [the town of Loja (Granada)] as opposed to other studied WWTPs serving much larger populations exceeding 320,000 and 700,000 inhabitants in the case of the cities of Córdoba or Sevilla, respectively. The elimination (degradation or transformation) of the target compounds after treatment in the

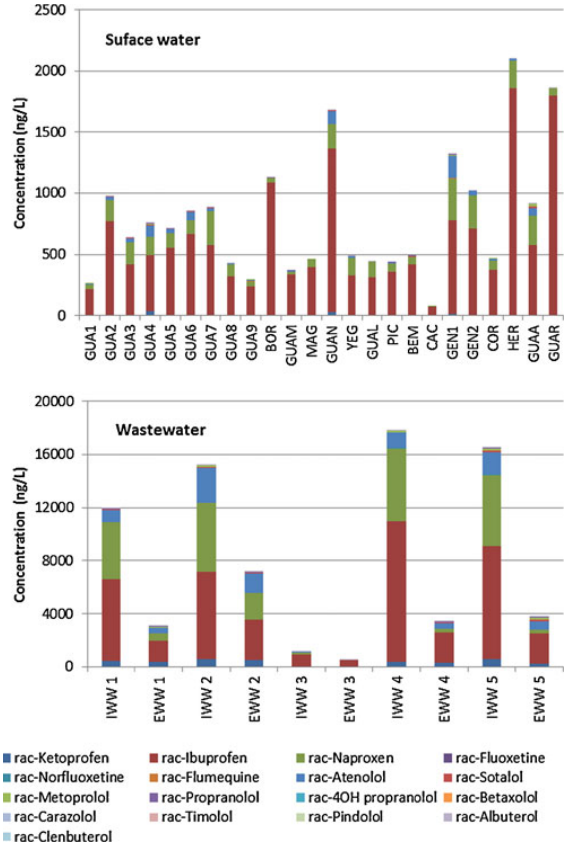

Fig. 5 Spatial distribution of chiral pharmaceuticals and their metabolites in the Guadalquivir River basin

monitored WWTPs was evident, with a decrease in the total concentration exceeding 50 %. The best performance was observed in the case of WWTP4 with a decrease in the cumulative concentration of the target compounds of 81 %.

The cumulative concentrations of studied analytes in receiving waters were fairly constant throughout the study and did not on average exceed 2,000 ng L^{-1}. Thus, GUA4, GUA6, GEN1 and GUAA sampling sites, which receive treated wastewater with high cumulative concentrations of studied pharmaceuticals, did not show any significant differences in concentrations when compared to other SWs. Such a situation can be attributed to an elevated dilution factor in the above sites. The cumulative concentration observed for SW samples was higher than 2,000 ng L^{-1} in only one sampling location, HER, in Herreros River, a tributary of Guadalquivir. In general, the highest concentrations were found in tributaries to the main river Guadalquivir, i.e. HER, GUAR, GUAN, GEN1, BOR and GEN2, all of them with cumulative concentrations above 1,000 ng L^{-1}. This phenomenon, which was also observed in previous studies in other rivers in the Iberian Peninsula [16, 17], is attributed to

the lower dilution factor due to the lower river flows found in the tributaries compared to the ones in the main River (see Table S2, Electronic Supplementary Material). In contrast, sites like GUA1, GUA9 and CAC showed the lowest contamination with total concentration below 300 ng L^{-1}. This is not surprising for GUA1 and CAC which correspond to locations near the source of the river. However, GUA9 was situated near the mouth of the main Guadalquivir River, and the low concentrations detected there reflect very high dilution factors due to high river flow in the final part of the river. In fact, the concentration remained quite similar along the main Guadalquivir River. This means that despite the input of effluents from the WWTPs of big cities (like Córdoba and Sevilla) situated along the river, the dilution phenomenon keeps the concentrations stable. Natural attenuation could also be taking place.

Conclusions

This paper presents the development and application of an analytical method for the determination of 16 chiral pharmaceuticals and two of their active metabolites in SW and wastewater (EWW and IWW) by LC–ESI-MS/MS. The method allowed for successful baseline resolution of 13 pairs of enantiomers of chiral drugs and partial separation of enantiomers of norfluoxetine. The comprehensive method development revealed that the Chirobiotic V stationary phase allowed only for the separation of basic compounds (with pK_a >8). In contrast, enantiomers of acidic analytes (with pK_a <5) were not resolved and eluted with very short retention times indicating weak interactions with the stationary phase.

The method validation revealed very good sensitivity in all matrices, with LOQs lower than 10 ng L^{-1} for most of the target analytes, even in wastewater. The reliability of the method was assured with the inclusion of 13 isotopically labelled surrogates.

A monitoring programme of the Guadalquivir River basin (South Spain), including 24 sampling sites and five WWTPs along the basin, revealed that the enantiomeric composition of the pharmaceuticals studied is dependent on compound and sampling site.

Several compounds such as ibuprofen, atenolol, sotalol and metoprolol were present generally as racemic mixtures. On the other hand, the enantiomeric profiles of fluoxetine, propranolol and albuterol showed a predominance of one enantiomer. Such an outcome might be of significant environmental relevance as two enantiomers of the same chiral compound might reveal different potency and ecotoxicity. For example, propranolol was enriched with S(−)-enantiomer. This could be a significant finding as S(−)-propranolol has been found to be more toxic to *P. promelas* [21]. Fluoxetine was found to be enriched with S(+)-enantiomer. Enrichment of fluoxetine with *S*(+)-enantiomer is of potentially significant ecotoxicological consequence as toxic effects of fluoxetine enantiomers are species dependent with *S*-fluoxetine being more toxic than *R*-fluoxetine in *P. promelas* [24].

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2.3 Discusión de resultados

Todos los métodos aquí expuestos, presentan como innovación el ser altamente multi-residuo. Incluyen un elevado número de fármacos, así como sus principales productos activos de transformación. En la Tabla A1 de los anexos, se presentan todos los compuestos incluídos en cualquiera de los cuatro métodos, junto con sus principales características físico-químicas. Son un total de 96 compuestos, y están distribuídos en grupos terapéuticos (14 analgésicos/anti-inflamatorios, entre ellos 3 TPs; 8 reguladores de lípidos, entre ellos 2 TPs; 13 drogas psiquiátricas, entre ellos 8 TPs; 5 antihistamínicos, entre ellos 1 TP; 31 antibióticos, entre ellos 5 TPs; 16 drogras cardiovasculares, entre ellos 3 TPs; 2 β-agonistas; 3 barbitúricos; 3 antidiabéticos, entre ellos 2 TPs; 1 antineoplástico). Es decir, 72 fármacos y 24 TPs. Los Métodos 1 y 2 permiten la deteminación de 74 residuos, fundamentalmente fármacos. En el Método 3, se añadieron 3 compuestos más, incluyendo un total de 77 entre fármacos y TPs. No obstante, la elevada sensibilidad del espectrómetro de masas utilizado en el Método 2 (Applied Biosystems Q-Trap) posibilitó la inclusión de otros 21 TPs, determinando así, 95 de los 96 compuestos aquí discutidos, como se discute más adelante en este mismo apartado, y se presenta en el capítulo 3 de la presente memoria, bajo la publicación científica 7. El Método 4 determina enantioméricamente 14 compuestos (28, si se considera cada enatiómero como una sustancia diferente), lo que supone un gran logro en el análisis quiral.

Todos los TPs incluidos corresponden a metabolitos humanos, excepto el acridin y el acridón que son subproductos de algunos tratamientos de degradación. No obstante, estos residuos pueden también ser generados durante el metabolismo animal, e incluso formarse en las EDARs o como consecuencia de transformaciones físico-químicas o biológicas en el medioambiente. La gran mayoría de los TPs aquí analizados son farmacológicamente activos (9), o potencialmente activos (8), es decir, derivados glucuronizados o acetilados de los fármacos, que han sido generados como estratategia para proporcionar polaridad a los fármacos, y así facilitar su excreción. Sin embargo, este grupo acetil o glucuronido está unido al fármaco por un enlace lábil, y por lo tanto, puede romperse con facilidad, por ejemplo, en las EDARs o ya en el medio ambiente, liberando el fármaco activo [81]. No obstante, además de la toxicidad persé que todos estos compuestos presentan debido a que se trata de sustancias producidas para que ejerzan una actividad biológica, no se puede excluir otras modos adicionales de acción ecotóxicológica [253, 254].

Esta tabla recoge también las estructuras y las principales propiedades físico-químicas (masa molecular, pKa y log P), que han sido útiles durante el desarrollo y optimización de los métodos, así como en el entendimiento e interpretación de los resulados obtenidos en los subsecuentes análisis. Más de la mitad (49) de los analistos aquí expuestos presentan quiralidad. Sin embargo, solo los que presentaban un único carbono quiral fueron incluidos en el Método 4. Un asterisco indica la posición de dicho C quiral en las estructuras de los compuestos incluidos en tal método. Se incluyen compuestos tan ligeros y pequeños como

el Metronidazole, con masa molecular 171.15, y tan voluminosos y pesados como el Tylosin, con masa molecular 916.1. El pKa, nos muestra que las sustancias aquí referidas presentan un extraordinariamente amplio intervalo de acidez, comprendido entre el -4.08 del Hydrochlorothiazide, y el 14.13 del Cimetidine. Del mismo modo, la lipofilia, representada por el log P, nos muesta la gran disparidad de polaridades representadas con estos compuestos, con un rango que se extiende desde -3,151 del Ranitidine-N-oxide y 5,801 del Fenofibrate. Esto pone de manifiesto la gran capacidad de los métodos multi-residuo desarrollados, y deja la puerta abierta a la introducción de nuevos compuestos, siempre que otros parámetros como la sensibilidad, lo permitan. Esto es así puesto que con mucha probabilidad, presentarán características dentro de estos intervalos, y por lo tanto, se esperarán comportamientos de los parámetros de validación similares a los aquí presentes. Así en el trabajo presentado en el capítulo 3 de la presente memoria, y descrito en la publicación científica 7 ("Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain" por Rebeca López-Serna, Anna Jurado, Enric Vázquez-Suñé, Jesus Carrera, Mira Petrović, Damià Barceló en "Environmental Pollution") se utilizó el Método 2, ligeramente modificado para la introducción de 21 de sus TPs.

En la última columna se indica el surrogate/patrón interno utilizado en el Método 1, 2, 3 o 4, respectivamente. Una raya (-) indica que el correspondiente método no incluye ese compuesto. El hecho de que para algunos compuestos no se haya utilizado el mismo surrogate/patrón interno en todos los métodos, se debe a que no en todos ellos se han incluido los mismos "chivatos", así como a que, al llevarse a cabo diferentes métodos cromatográficos, la asignación óptima analito-surrogate/patrón interno, varia.

Por otro lado, la Tabla 4, resume y compara las principales características de los cuatro métodos aquí discutidos. Todos ellos pueden ser divididos en 6 etapas: 1) filtración, 2) adicción de sustancias I, 3) pre-tratamiento, 4) addicción de sustancias II, 5) separación por cromatografía de líquidos, y 6) técnica de detección por espectrometría de masas. No obstante, algunos métodos prescinden de algunas de las etapas de adicción de sustancias. Así por ejemplo, en los métodos 2, 3 y 4, todos los aditivos, incluidos los surrogates isotópicamente marcados, se han añadido en la etapa previa al pre-tratamiento, desapareciendo la etapa de adicción de sustancias II, donde se añaden los patrones internos en el Método 1. En este último método, los chivatos solo van a dar cuenta de las incidencias producidas durante la detección, es decir, fundamentalmente van a corregir el efecto matriz durante la ionización por ESI. La corrección de las deficiencas en los porcentajes de recuperación durante el pre-tratamiento se lleva a cabo tras calcular dichos porcentajes sobre alícuotas de muestras dopadas a concentración conocida sometidas al mismo método analítico. No se añadieron los chivatos durante la etapa de adicción de sustancias I, puesto que el número de patrones isotópicamente marcados no era suficientemente elevado (23 patrones específicos isotópicamente marcados para 74 compuestos) como para usarlos como surrogate y corregir la efectividad del método de manera segura. La situación ideal es

la dilución isotópica, es decir, contar con un patrón específico isotópicamente marcado para cada compuesto incluido en el método. En cualquier caso, la corrección del efecto matriz durante la ESI es fácilmente llevada a cabo siempre que el chivato utilizado sea similar estructuralmente (ejemplo, pertenciente a la misma familia) y presente un tiempo de retención cromatógrafico similar al analito al que corrige. Sin embargo, para llevar a cabo una corrección de confianza de las deficiencias durante el pre-tratamiento, se requiere una semejanza mucho más exigente entre el chivato, y el analito a corregir. Es por esto, que solo en situaciones cercanas a la dilución isotópica (Método 2, 50 patrones específicos isotópicamente marcados para 74; Método 3, 46 patrones específicos isotópicamente marcados para 77; Método 4, 11 patrones específicos isotópicamente marcados para 17), estos chivatos se han añadido durante la adicción de sustancias I. Por lo tanto, los Métodos 2, 3, 4, son métodos más ventajosos, donde se han eliminado etapas, simplificado la cuantificación, y además, son más fiables, por la seguridad que proporcionan tal número de patrones específicos isotópicamente marcados. Otros aditivos añadidos durante la adicción de sustancias I, para mejorar el rendimiento de la etapa de pre-tratamiento, son agente complejantes, como el EDTA en los Métodos 1 y 2, agentes desorbentes de los analitos sobre el vidrio del material fungible utilizado, así como ácidos o bases para ajustar el pH. Sin embargo no existe una pauta común con respecto al tipo de pre-tratamiento a utilizar, ni siquiera cuando se emplean los mismos sorbentes. Los Métodos 1, 2 y 4 llevan a cabo pre-tratamientos por SPE, el primero y el último emplean Oasis HLB como fase estacionaria, y sin embargo, los aditivos añadidos durante adicción de sustancias I difieren.

El volumen de muestra necesario para el análisis depende en primer lugar, de si se hace uso de un pre-tratamiento online (Métodos 2 y 3) u offline (Métodos 1 y 4), es decir, automatizado y en línea con la cromatografía de líquidos y la espectrometría de masas, o no. En el primer caso, el requerimiento es de unidades de mL, mientras que en el segundo es de centenas de mL, lo que puede suponer problemas de almacenamiento, cuando el número de muestras a analizar aumenta. Además, en este último caso, existen también diferencias en el volumen de muestra necesario en función de la naturaleza de la matriz. Así, para aguas complejas y presumiblemente más concentradas, como las aguas residuales de entrada (IWW, *Influent Waste Water*) y de salida (EWW, *Effluent Waste Water*) de las EDARs, se requieren tamaños de muestra inferiores, a los de aguas superficiales (SW, *Superficial Water*) y subterráneas (GW, *Groundwater*).

Todos los métodos aquí presentados incluyen una etapa de separación de los analitos por cromatografía de líquidos analítica, previa a la detección. Para los Métodos 2, 3 y 4, ésta consiste en HPLC, haciendo uso de columnas analíticas con tamaño de partícula superior a 2 μm. Sin embargo, en el Método 1, se empleó una columna con tamaño de partícula 1.7 μm, operando así en UHPLC, lo que supuso un acortamiento ostensible en los tiempos cromatográficos, lo que repercutió postivamente en los tiempos de análisis por muestra. En los Métodos 2 y 3, se hicieron pruebas para incluir cromatografía rápida. En el Método 2 se probó una columna fused-core, obteniendo buenos resultados cromatográficos, pero el

detector Applied Biosystems Q-Trap que se caracteriza por su excelente sensibilidad, resultó sin embargo, no ser suficientemente rápido para registrar suficientes puntos en los estrechos picos generados. En el método 3, las bombas cromatográficas utilizadas (Thermo Fisher Scientific Accela LC system), son capaces de soportar presiones de hasta 600 bares, y el detector empleado (Thermo Fisher Scientific TSQ Vantage), presenta una velocidad de adquisición suficientemente rápida como para soportar una cromatografía rápida. Así, se probaron flujos elevados y columnas cromatográficias analíticas con tamaños de partículas sub-2 μm, trabajando en cuasi-UHPLC, pero ninguna de estas opciones proporcionaban perfiles cromatográficos con picos gausianos adecuadas, por lo que se desestimó su uso. En el Método 4, se utilizó un equipamiento similar al utilizado en el Método 1 (cromatógrafo Waters Acquity UPLC, capaz de soportar presiones de hasta 1000 bares, y un detector Acquity Xevo TQD con velocidad de adquisición adecuada para la proporcionanr suficiente puntos en un pico estrecho generado por cromaografía rápida). Sin embargo, se observó que al aumentar la velocidad de flujo de la fase móvil, la separación enantiomérica empeoraba, por lo que el método cromatográfico optimizado consistió en un gradiente isocrático de 66 min, que generaba una presión de trabajo en torno a los 30 bares. No obstante, las especificaciones de la columna quiral utilizada (Chirobiotic V) indican que esta fase estacionaria no debe ser sometida a presiones superiores a 240 bares. Para los Métodos 1, 2 y 3, los métodos cromatográficos, consistían en gradientes.

La técnica de detección fue, en todos los casos, la MS/MS en modo SRM, utilizando la ESI como interfase. En los métodos 1, 2 y 3 se adquirió en ambos modos positivo (ESI+) y negativo de ionización (ESI-), mientras que en el Método 4, que solo incluyó 17 residuos, fue suficiente registrar en ESI+ todos los compuestos. Éste ultimo método, y por la misma razón, no necesitó el uso de ventanas de adquisición para proporcionar una sensibilidad de método suficiente. Si se establecen, las ventanas de adquisición, tienen que ser lo más cortas posibles, para que su efectividad sea máxima, pero al mismo tiempo, suficientemente largas como para proporcionar seguridad en el registro de los compuestos, puestos que sus tiempos de retención pueden variar, especialmente cuando se analizan matrices de complejidad variable (ejemplo, GW y sewage water). Por esta razón, se recomienda hacer uso de las ventantas de adquisición, solo cuando sean estrictamente necesarias para conseguir límites de detección adecuados para determinar este tipo de microcontaminantes en estas matrices medioambientales. Este fue el caso del Método 1, en el que a pesar de utilizar un detector de similares características (Waters Acquity TQD) al Método 4, fue necesario establecer ventanas de adquisición, puesto que el número de transiciones a registrar en el primero (dos por cada uno de lo 74 compuestos, más una por cada uno de los 23 patrones internos) era casi cuatro veces mayor. Similar número de transiciones o incluso superior, fueron registradas con los métodos 2 y 3. Sin embargo, el detector del Método 2, el Applied Biosystem Q-Trap, es tan sensible que es capaz de alcanzar límites de detección de pocos ng L^{-1} adquiriendo en una sola ventana, semejante cantidad de transiciones. En los cuatro métodos, la cuantificación se lleva a cabo por patrón interno, haciendo uso de los

chivatos isotópicamente marcados, ya hayan sido añadidos antes o después de la etapa de pre-tratamiento. Si bien, como se ha comentado anteriormente en este mismo apartado, para el caso del Método 1, se requiere, además, una corrección adicional de la eficiencia durante el pre-tratamiento.

A continuación vamos a valorar las bonanzas y deficiencias de los métodos desarrollados, en función de cinco propiedades: fiabilidad, versatilidad, rendimiento, sensibilidad y capacidad. Buenos indicadores de fiabilidad son el tipo y número de "chivatos" (patrones internos o surrogates) incluidos en el método en relación con los analitos. Los Métodos 2, 3 y 4, usan los chivatos como surrogates, es decir, son añadidos antes de la etapa de pre-tratamiento, por lo que controlan la práctica totalidad del procedimiento, compensando cualquier incidencia que se pueda producir. A diferencia, el Método 1 puesto que añade los chivatos al final del método, antes de la LC-MS/MS. Las deficiencias durante la etapa de pre-tratamiento se corrigen mediante el cálculo, sobre muestras dopadas a concentración conocida, de los porcentajes de recuperación durante dicha etapa. Sin embargo, esta práctica, además de tediosa, no corrige de posibles incidencias individuales que tengan lugar sobre las muestras, como pérdida parcial de muestra por derrames, etc. En todos los métodos, los chivatos utilizados corresponden a derivados específicos isotópicamente marcados de analitos incluidos en el método. Esta es siempre una elección preferida frente a la utilización de compuestos análogos, por la certeza de que no se detectan en las muestras, y por la total similitud en comportamiento físico-químico con su correspondiente analito sin marcar. El Método 2 seguido del 4, son los que incluyen un mayor número de chivatos específicos isotópicamente marcados con respecto al número de analitos (68% y 65%, respectivamente). De nuevo, el Método 1, es el que proporciona menores porcentajes (31%), y es que, a pesar de disponer de más patrones específicos isotópicamente marcados, el método no admitía más transiciones SRM, debido a limitaciones de sensibilidad del detector utilizado (Waters Acquity TQD). Además, se trata de un método con baja automatización, puesto que la etapa de pre-tratamiento se lleva a cabo de manera manual y desacoplada a la LC-MSMS. La automatización es considerada otro indicador importante de fiabilidad, puesto que disminuye la probabilidad de errores por manipulación, y en la mayoría de los casos, simplifica el proceso, eliminando etapas del método. Los Métodos 2 y 3 están altamente automatizados, con las etapas de pre-tratamiento-LC-MS/MS acopladas en línea. Así, tras toda esta discusión, se puede dictaminar que el Método 2 es el más fiable de los cuatro.

Los Métodos 1 y 2 han sido validados satisfactoriamente para cuatro matrices medioamentales, GW, SW, EWW y IWW. El Método 2, además, fue utilizado para el análisis de agua potable, puesto que la complejidad de la matriz era menor, y la sensibilidad del método suficientemente potente para determinar sus niveles de ultra-traza. El Método 4 fue validado solo para tres de estas matrices (SW, EWW y IWW), si bien, cabe esperar que sea apto también para GW e incluso DW, por la buena sensibilidad que el método presenta. Por otro lado, ya se comentó en la publicación científica 3 incluída en el presente capítulo,

que el Método 3 presenta problemas durante su aplicación sobre matrices medioambientales complejas como las aguas residuales, puesto que la técnica de clean-up utilizada durante el pre-tratamiento (TurboFlow), no es eficiente eliminando los componentes propios de las matrices medioambientales, como los ácidos húmicos, generando inasumibles problemas de efecto matriz, además de una obturación excesiva y exponencial del detector, lo que condujo a todo tipo de incapacidades, como riesgo de degeneración de secciones esenciales en el espectrómetro de masas, avocamiento a tareas de limpieza con excesiva frecuencia, caída brutal de sensibilidad, disminución de la fiabilidad, etc. Por todo lo anterior, se considera que el Método 2 es, de nuevo, el más versátil.

La Figura 3, compara el tiempo de análisis por muestra de cada uno de los cuatro métodos aquí presentados frente a un "método convencional". Se ha escogido como convencional el método descrito por [255], puesto que determina un número y tipo similar de residuos y no incluye ninguna de las innovaciones aquí presentadas (UHPLC, pre-tratamiento online, análisis enantiomérico, etc.). Este tiempo de análisis por muestra es aproximado, pero nos da idea, del acortamiento aportado por las innovaciones que cada método incorpora. Los Métodos 2 y 3 son los que menos tiempo de análisis por muestra necesitan, indicando que el uso o no de técnicas de pre-tratamiento automatizadas, y sobre todo, acopladas a las etapas de separación y detección, es el parámetro que más influye en el rendimiento del método. Además, a mayores de un acortamiento en el tiempo de análisis por muestra, los métodos automatizados, permiten un mayor aprovechamiento del tiempo, puesto que pueden trabajar desatedidos en ausencia del analista, por ejemplo por la noche. El Método 4, sigue un diagrama de flujo muy similar al método convencional. Así la estimación de tiempo de análisis por muestra es semejante, incluso a pesar de que el número de residuos incluidos en el Método 4 es muy inferior (17 frente a 73 en método convencional) y de que una sola inyección por muestra en modo ESI+ es necesario. Esto es debido al largo método cromatográfico necesario (66 min) para conseguir una óptima separación enantiomérica. El Método 1, a pesar de reducir drásticamente el método cromatográfico con la utilización de la tecnología UHPLC, el tiempo total por muestra no se ve mermado tan ostensiblemente como en los Métodos 2 y 3, puesto que la etapa limitante es el pre-tratamiento de muestra, que es offline en el caso del Método 1. Por lo tanto, el método más ventajoso en términos de rendimiento es otra vez el Método 2, que con respecto al Método 3, consume menos tiempo durante la adicción de sustancias I, puesto que en éste último incluye más aditivos, y además debe realizarse dos veces por muestra (es decir, prepararse dos alícuotas diferentes por muestra), puesto que el ajuste de pH para el análisis por ESI+ y – difiere.

En cuanto a sensibilidad, y escogiendo SW como matriz comparadora, puesto que es la única por todos validada, los límites de detección obtenidos han sido inferiores a 0,1 ng L^{-1} para algunos de sus compuestos en todos los métodos. Pero solo el Método 4 consigue LODs inferiores a 10 ng L^{-1} para todos sus analitos. Esto se debe, muy posiblemente, al pequeño número de compuestos determinados por este método. Comparando los otros

tres métodos restantes, que analizan una cantidad similar de residuos, el Método 2 es el que resulta ser más sensible, presentando LODs para todos sus compuestos por debajo de 24 ng L^{-1}.

La capacidad de proporcionar información, entendida como la cantidad de sustancias que pueden ser determinadas por un solo método, deja al Método 4 muy en desventaja, con un total de 17 residuos incluídos. Si bien, el número real ascendería a 31, si se considera a cada enantiómero como un compuesto diferente. En cualquier caso, la capacidad de los Métodos 1, 2 y 3, con 74, 74 y 77 residuos cada uno, respectivamente, es claramente superior. Además, la capacidad del Método 2 se aumentó posteriorment (publicación 7 incluida en el capítulo 3 de la presente memoria) añadieron 21 TPs más, elevando a 96 el número de analitos. Este incremento en la capacidad, es siempre bienvenido en los estudios medioambientales, para obtener información acerca de la presencia acumulada de varios grupos de analitos en un lugar y momento determinados, y poder considerar así, por ejemplo, efectos toxicológicos sinérgicos sobre la vida acuática [141]. En este caso, posibilita la comparación de las concentraciones de fármacos y algunos de sus TPs de más interés, con la aplicación de un solo método, con el ahorro de tiempo, recursos y esfuerzos que esto supone.

Por último, haciendo una valoración sobre el impacto medioambiental, el Método 2 es de nuevo el que ocupa la posición más favorable. Debido a que se trata de un método altamente automatizado y acoplado, y a que requiere el menor tamaño de muestra (5 mL), el gasto de disolvente y aditivos asociado en la preparación de disoluciones y lavado del material utilizado, es también el menor. El Método 3, a pesar de ser también online y de que requiere solo 2.5 mL de muestra más, el gasto de disolvente necesario se dispara debido a que el inyector de muestra utilizado está diseñado de manera que requiere una exhaustiva limpieza post-inyección a base de disolvente, si se quieren evitar episodios de carry over. Además el sistema de preparación de muestra TurboFlow precisa de flujos de fase móvil muy elevados, por lo que el consumo de disolvente también lo es. Por otro lado, la técnica de TurboFlow emplea columnas de larga vida, diseñadas para que puedan ser usadas para el pretratamiento de muestra de entre 500-1000 inyecciones, en contraposición a la SPE, donde la reutilización de los cartuchos se desaconseja. Un menor uso de disolventes y aditivos, no solo redunda en una coherencia ética presumible de una herramienta medioambiental como los son estos métodos, sino también, en menor riesgo laboral para el analista durante la utilización del método.

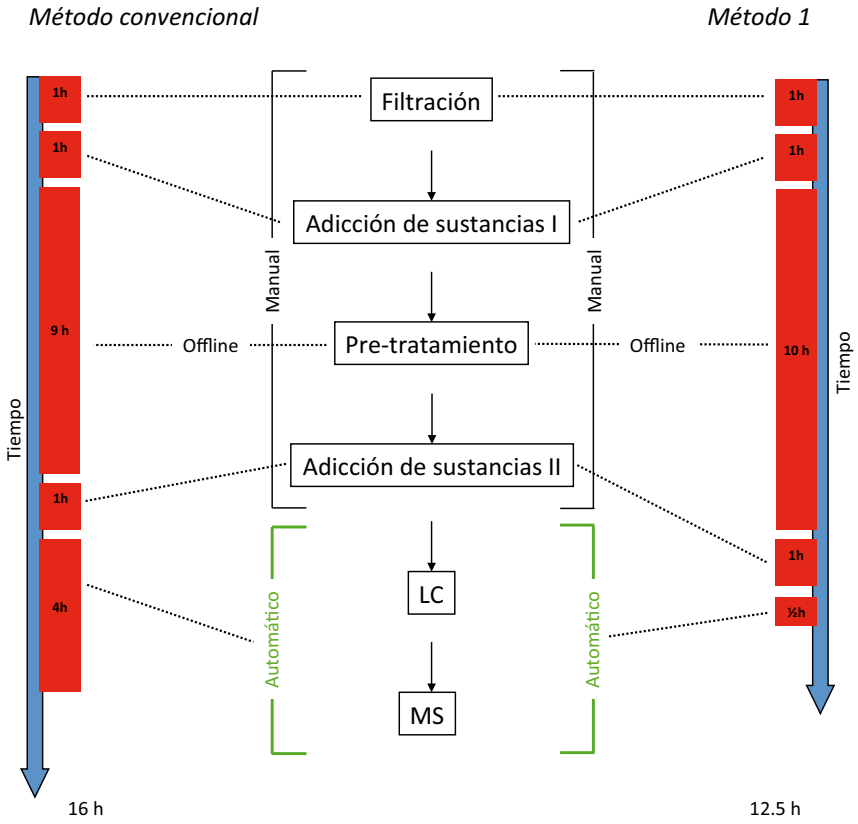
De todo lo anterior se extrae, que el Método 2 ha resultado ser el más completo de los cuatro, el que más posibilidades presenta y el que más bonanzas auna. Si bien, no es perfecto. Y así, uno de sus principales puntos fuertes, la sensibilidad, es proporcionada fundamentalmente por el potente detector que utiliza, el Applied Biosystems Q-Trap, el cuál al mismo tiempo, sin embargo, presenta la carencia de presentar una velocidad de adquisición escasa, lo que limita su uso con cromatografías rápidas. Tampoco, y por la

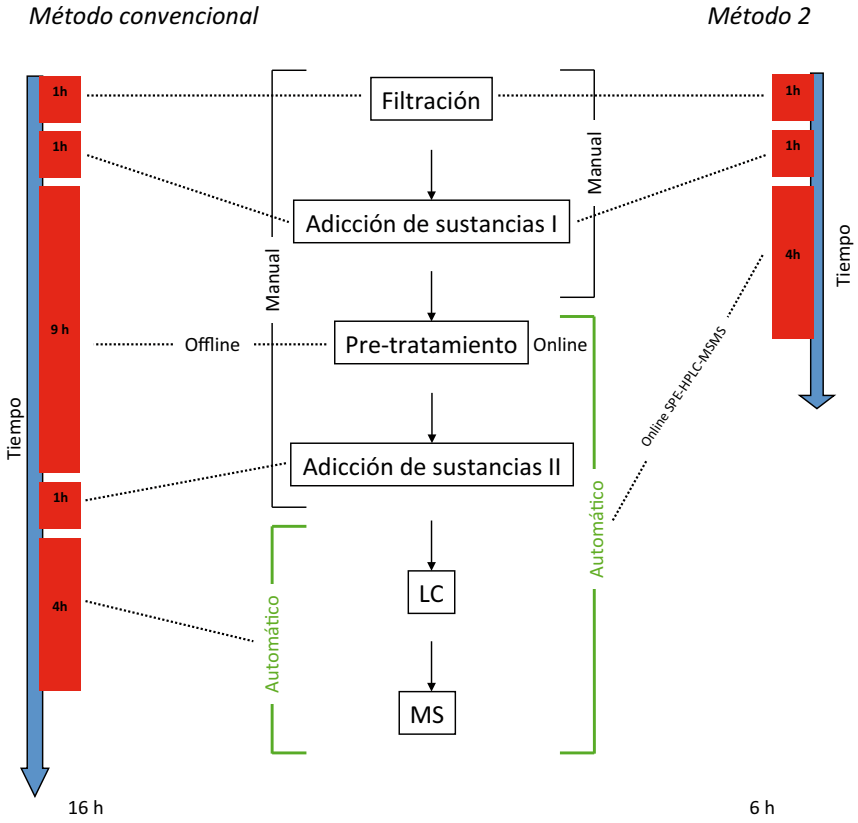
Tabla 4. Resumen de características principales de los cuatro métodos desarrollados Método 1, 2, 3 y 4.

	Método 1	Método 2	Método 3	Método 4
Filtración				
Volumen de muestra (GW, SW, EWW, IWW), mL	500, 500, 200, 100	5, 5, 5, 5	7.5, 7.5, 7.5, 7.5	-, 500, 100, 100
Adicción de sustancias I				
Adicción de aditivos	EDTA	EDTA	MeOH	-
Ajuste de pH	-	-	ESI+: 0.1% FA + 0.02% TFA / ESI-: 10 mM NH4Ac	7.5
Addicción de *surrogates*	-	51	46	11
Pre-tratamiento				
Tipo de pretratamiento	Offline SPE	Online SPE	Online TurboFlow	Offline SPE
Fase estacionaria en el pretratamiento	Oasis HLB	HySphere Resin GP	Cyclone P, C18-P XL, Cyclone MAX	Oasis HLB
Adicción de sustancias II				
Adicción de patrones	24	-	-	-
Cromatografía de líquidos				
Técnica cromatográfica	UHPLC	HPLC	HPLC	HPLC quiral
Columna	Waters Acquity UHPLC BEHC18 (100 mm x 2.1 mm, 1.7 µm)	Merck Purospher Star RP-18 endcapped (125 mm x 2.0 mm, 5 µm)	Thermo Fisher Scientific Betasil Phenyl-Hexyl (50 mm x 3 mm, 3 µm)	Astec Chirobiotic V (250 x 2.1 mm, 5 µm)
Tipo de elución	Gradiente	Gradiente	Gradiente	Isocrático
Duración del método cromatográfico, min	5 (ESI-) + 8 (ESI+)	30 (ESI-) + 37 (ESI+)	21.48 (ESI-) + 22.42 (ESI+)	66 (ESI+)

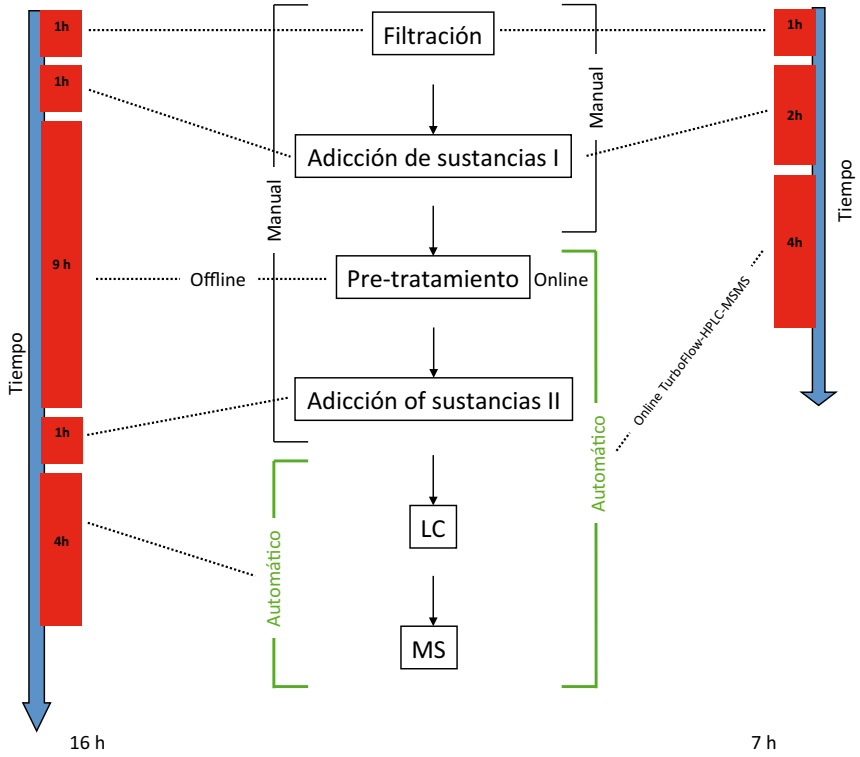
Espectrometría de masas				
Interfase	ESI	ESI	ESI	ESI
Polaridad	+/-	+/-	+/-	+
Tipo de analizador	MS/MS	MS/MS	MS/MS	MS/MS
Modo de adquisición	SRM	SRM	SRM	SRM
Método de cuantificación	Patrón interno	Patrón interno	Patrón interno	Patrón interno
Ventanas de adquisición	S	N	S	N
Validación				
Fibilidad				
Tipo de "chivato"	IS	Surrogate	Surrogate	Surrogate
Nº "chivatos" isotópicamente marcados	23	50	46	11
Nº compuestos	74	74	77	17
Automatización	Baja	Muy alta	Alta	Baja
Versatilidad				
Tipo de matrices estudiadas	GW, SW, EWW, IWW	GW, SW, EWW, IWW	GW, SW	SW, EWW, IWW
Rendimiento				
Tiempo total de análisis/muestra, horas (ver Figura 3)	12.5	6	7	15
Sensibilidad				
Rango de límites de detección del método en SW, ng L^{-1}	0,02 - 53,97	0,01 - 23,49	0,03 - 49,3	0,09 - 3,47
Capacidad				
Nº compuestos (Nº fármacos + Nº TPs)	74 (72+2)	74 (72+2)	77 (58+19)	17 (15+2)
Otros				
Gasto de disolvente / fungible	Medio	Mínimo	Alto	Medio

Figura 3. Comparación del rendimiento de cada método desarrollado frente a un método convencional

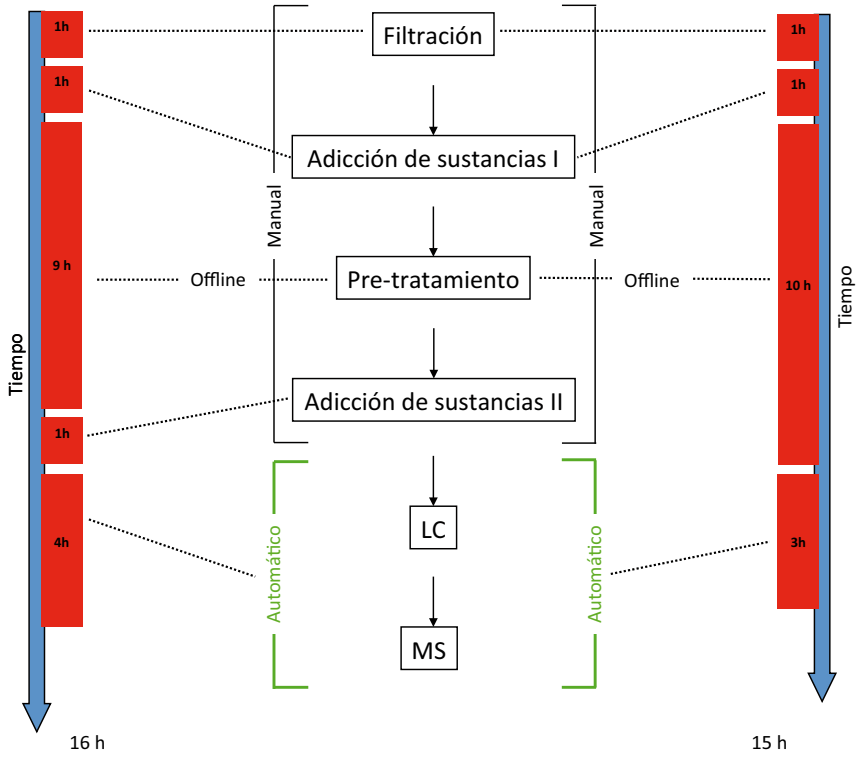




Método convencional *Método 3*



Método convencional *Método 4*



misma razón, es capaz de trabajar en modo ESI+ y – en una misma inyección. Ambos dos atributos redundarían, si los poseyera, en un tiempo de análisis por muestra aún más corto, es decir, en un rendimiento mayor. Esto, junto con una situación de dilución isotópica total, lo convertirían en un método de extraordinarias capacidades.

3. ESTUDIOS DE PRESENCIA DE FÁRMACOS EN AGUAS MEDIOAMBIENTALES

3.1 Introducción

La existencia de drogas en las aguas medioambientales fue por primera vez publicada en los años ’70 por Norpoth et al. [256], entre otros; y los primeros estudios informando la existencia de drogas en las aguas residuales se remontan a los años ’80 [257, 258]. Desde entonces, numerosos estudios han confirmado la existencia de compuestos farmacéuticos en matrices acuáticas, sedimentos, suelos y fangos.

La investigación de drogas en aguas superficiales ha sido intensa en los últimos años [39]. El menor número de estudios relacionados con aguas residuales se atribuye a la complejidad de la matriz y al hecho de que solo un pequeño número de laboratorios tenía hasta hace poco, la capacidad de llevar a cabo tales análisis usando equipos y métodos sofisticados de análisis químicos.

Existen varias revisiones bibliográficas publicadas en la literatura científica, informando de la presencia de diferentes fármacos en el medio ambiente acuático. No obstante, se hace complicado la comparación de niveles, y de hecho, fluctúan sustancialmente incluso comparando mismas matrices (GW, SW, EWW, IWW, etc.). Esto debido a que son muchos los factores que pueden influir. Algunos de ellos, con respecto a las aguas medioambientales, se indican a continuación:

- Hábitos de consumo de fármacos en la zona
- Método de muestreo y analítico utilizado
- Eficiencia de los tratamientos de las EDARs con influencia en el sistema hídrico
- Cercanía del punto de muestreo a un punto de vertido de una EDAR
- Stress antropogénico: Influencia de importantes núcleos de población
- Caudal / Efecto de dilución
- Pluviosidad y climatología general de la zona
- Tipo de geología de zona (composición de los sedimentos y el suelo)

A continuación se indican algunas de las conclusiones extraidas en algunas de las revisiones bibliográficas más recientes:

Fatta-Kassinos y colaboradores [39] revisaron en 2011 la presencia de fármacos en aguas medioambientales y residuales a nivel mundial y encontraron que las clases de fármacos más frecuentemente detectadas son los analgésicos y anti-inflamotorios, los antibióticos, los reguladores de lípidos, los beta-bloqueantes y los fármacos de tratamiento para el cáncer [259]. Dentro de estos, la carbamacepina, el diclofenaco, el ibuprofeno, el gemfibrocilo, el atenolol, el propranolol, la eritromicina, el ciprofloxacino, el ofloxacino, el sulfametoxazol y la amoxicilina, son algunos de los compuestos para los que los estudios presentan una presencia más extensa en el medio ambiente acuático, incluyendo el agua residual.

Tabla 5. Presencia en agua subterránea, superficial y residual urbana, de una selección de antibióticos

	Efluentes de EDARs urbanas		Aguas superficiales		Aguas subterráneas	
	Concentración (ng L^{-1})	Referencia	Concentración (ng L^{-1})	Referencia	Concentración (ng L^{-1})	Referencia
Acitromicina	4-23 / 75 / 15	[176, 260, 261]	-	-	-	-
Cefalexin	250 / 10-994 / 170-5070 / 283 / 240-1800 / 376	[260, 262-265]	100	[262]	-	-
Ciprofloxacino	720 / 240 / 132 / 627 / 140 / 400 / 8-73 / 220-450 / 62-106 / 108 / 251 / 42-392	[65, 115, 176, 260, 266-272]	1300 / 17.4-588.5 / <10 / 370-9660 / 14.4-26.2	[262, 267, 271, 273, 274]	-	-
Clorotetraciclina	250 / 50-280	[262, 275]	600 / 160 / 1-180	[262, 275, 276]	-	-
Claritromicina	536 / 172 / 240 / 70-611 / 57-328 / 100 / 12-232 / 18	[114, 176, 260, 265, 267, 271, 277, 278]	3-114.8 / 600-2330 / 190 / 1.6-20.3	[117, 267, 271, 274]	-	-
Doxiciclina	60-340 / 150 / 46 / / 40	[65, 114, 262, 275]	50-80 / 13-146 / 400	[262, 275, 276]	-	-
Enrofloxacino	50 /10	[65, 262]	300	[262]	-	-
Eritromicina	361-811 / 300 / 838 / 246-4330 / 695 / 510-850 / 38-96 / 110-199	[260, 263, 264, 268], [114, 265, 278]	4.7-1900 / 636	[263, 279]	-	-
Ácido nalidixico	-	-	750 / <10	[262, 273]	-	-

Norfloxacino	250 / 210 / 5.5-3700 / 85-320 / 112 / 120 / 36-73 / 64	[82, 114, 115, 262-264, 266, 270]	1150 / 163 / 251 / 24-48	[262, 273, 279, 280]	-	-
Ofloxacino	183 / 53-991 / 506 / 110 / 96-7870 / 123 / 2-556 / 50-210 / 600 / 32-548 / 740-5700	[114, 119, 260, 263, 265, 267, 271, 272, 281, 282]	19.3-306.1 / 8.1-634 / 55 / 108 / 33.1-306.1	[263, 267, 271, 274, 279]	-	-
Oxitetraciclina	70 / 20 / 100-340 / 5-842 / 5	[65, 262, 263, 275, 283]	100 / 7.7-105,1 / 80-130 / 1-388 / 110-680 / 2-7 / 68000	[262, 267, 274-276, 283, 284]	-	-
Roxitromicina	500 / 18 / 85-547 / 3 / 3-14 / 11-22 / 18	[65, 114, 260, 262, 263, 278]	350 / 169 / 190 / <30-40	[117, 262, 279, 285]	-	-
Sulfadiacina	6 / 16 / 34.3 / 19 / 4180	[260], [114], [282], [286]	1.9-316.8 / 336	[279, 286]	-	-
Sulfametacina	130-640 / 363 / 2 / 11 / 400	[114, 260, 275, 286]	220 / <10 / 1.7-6192	[273, 275, 286]	360 / 76-215 / 0.03-106.8 /	[287-289]
Sulfametoxazol	200 / 320 / 79-472 / 130-500 / 47-964 / 370 / 871 / 310 / 5-278 / 226 / 4-39 / 15-47 / 242 / 220-680 / 2000 / 289 / 127 / 132 / 9460	[65, 176, 260, 262, 263], [114, 119, 265, 268, 269, 271, 275, 277, 281-283, 286, 290]	2000 / 50-120 / 300 / 1-7 / 480 / 193 / <30-70 / 544 / 1-22 / 6.4-1488 / 47-96 /	[115, 117, 119, 262, 275, 276, 279, 280, 283, 285, 286]	9.9 / 1110 / 0.08-312.2	[286, 287, 289]

Tetraciclina	20 / 30 / 31-34 / 190-360 / 16-38 / 850 / 977 / 3.5-1420 / 21 / 150-620 / 24 / 89 / 61-290	[65, 114, 260, 262-265, 268, 269, 275, 277, 283]	80 / 60-140 / 1-5 / 7-8	[262, 275, 276, 283]	-	-
Trimetoprim	250 / 70 / 2-37000 / 203-415 / 550 / 180 / 59-465 / 321 / 120-230 / 11-66 / 210-2400 / 1070 / 1288 / 105 / 140	[65, 119, 176, 260, 262-264, 268, 269, 281, 290-292]	150 / <30-40 / 31 / 87 / 120	[117, 262, 273, 280, 285]	-	-
Tilosin	3400 / 65 / 7	[65, 260, 262]	60	[262]	-	-

En la Tabla 5, se recogen datos de presencia en agua subterránea, superficial y residual urbana, de algunos de los antibióticos incluidos en esta revisión bibliográfica, muchos de ellos, estudiados en la presente tesis.

En esta tabla se hace patente las importantes fluctuaciones observadas entre estudios, sobre matrices de la misma categoría, dando lugar a rangos amplios de presencia posible. Así por ejemplo, encontraron que el ciprofloxacino variaba, en efluentes de depuradora, entre 8 y 720 ng L^{-1}, el cefalexin entre 10 y 5070 ng L^{-1}, la eritromicina entre 38 y 4330 ng L^{-1}, y el sulfametoxazol entre 4 y 9460 ng L^{-1}. En aguas superficiales, el ciprofloxacino varió entre 14.4 y 9660 ng L^{-1}, el ácido nalidixico entre <10 y 750 ng L^{-1} y la claritromicina entre 3 y 2330 ng L^{-1}.

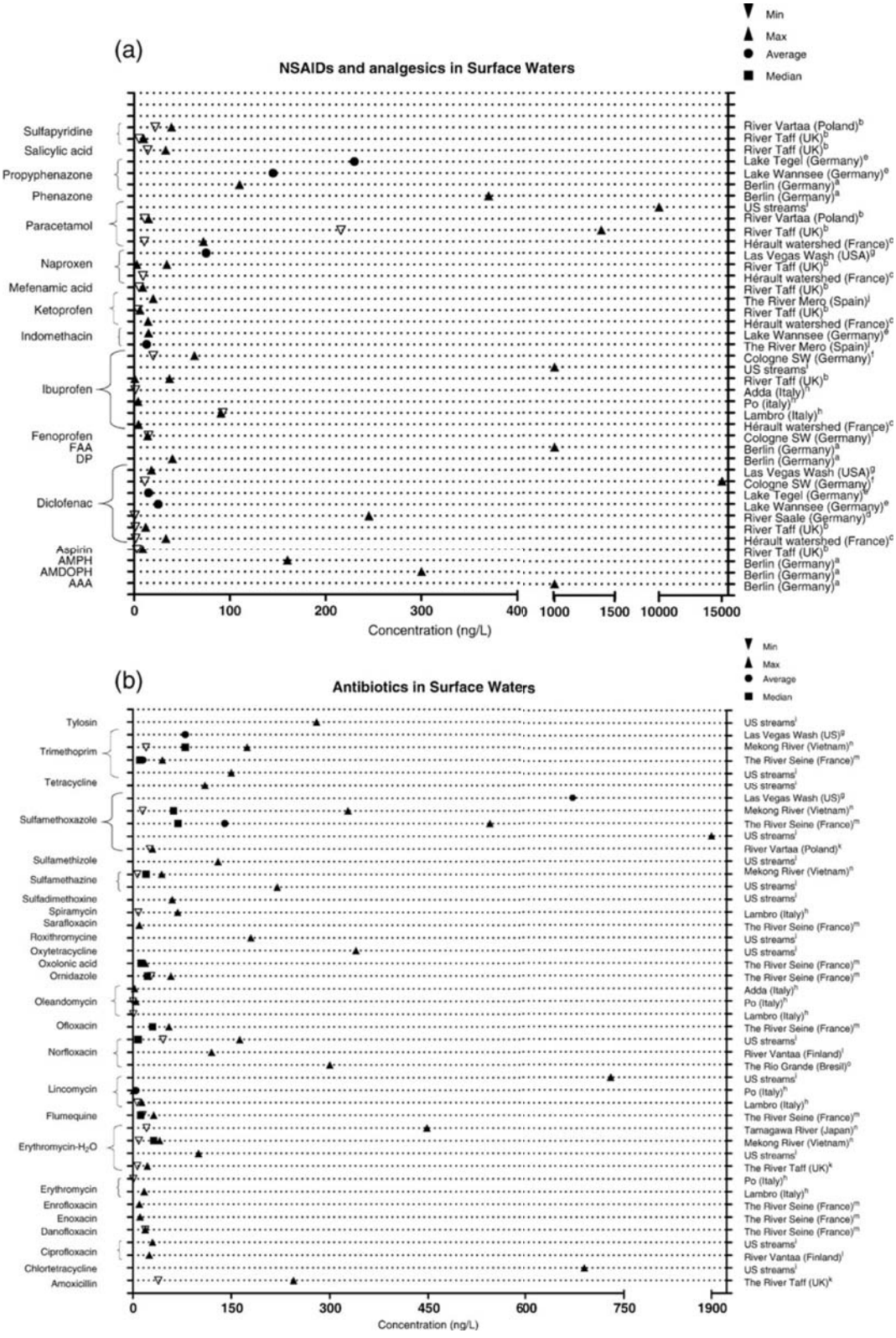
Otro trabajo de revisión importante fue el llevado a cabo en 2009 por Mompelat y colaboradores [36], que incluye además de fármacos, productos de transformación en aguas subterráneas y superficiales de todo el mundo.

Los estudios citados en esta revisión bibliográfica, atestiguan la presencia general de fármacos en las masas de agua desde ng L^{-1} hasta varios µg L^{-1}. Los autores observaron que son alrededor de 90 fármacos y productos de transformación los más comúnmente analizados, estando los TPs en menor proporción que los compuestos padre. Éstos pertenecían fundamentalmente a las clases terapéuticas de analgésicos y anti-inflamatorios y analgésicos. De nuevo observaron que fármacos del primer tipo eran los que presentaban las mayores concentraciones en las aguas superficiales, variando entre 0.4 ng L^{-1} y 15 µg L^{-1}, siendo el diclofenaco, el paracetamol y el ibuprofeno los que se cuantificaban a mayores concentraciones [293, 294]. Otras sustancias que consideraron importantes por su elevada presencia fueron el antibiótico sulfametoxazol, con µg L^{-1} en los EEUU [295], el anticonvulsivo carbamacepina cuantificado hasta 1.3 µg L^{-1} en Alemania [296] y en Canada [297], el regulador de lípidos gemfibrocilo con niveles de hasta 790 ng L^{-1} en EEUU [295], el anti-ácido ranitidina con concentraciones hasta 580 ng L^{-1} también en EEUU [295], el beta-bloqueante atenolol determinado a 241 ng L^{-1} en Italia [298] y el anti-diabético metformina con valores de hasta 150 ng L^{-1} en EEUU [295].

La Figura 4 muestra el mínimo, el máximo, la media y la mediana de los niveles encontrados para las fármacos pertenecientes a las familias de analgésicos y anti-inflamatorios no esteroides y antibióticos en los estudios de aguas superficiales incluidos en esta revisión.

Considerando el origen de los fármacos y los TPs en las aguas medioambientales, su concentración disminuye, obviamente, de las aguas residuales a las medioambientales. Así por ejemplo, los autores de esta revisión observaron que el ofloxacin, un antibiótico de la familia de las fluoroquinolonas, fue detectado a una concentración de hasta 35,5 µg L^{-1} en efluentes de hospitales en Alburquerque (Nuevo Méjico, EEUU), y a concentraciones de 410 ng L^{-1} y 110 ng L^{-1} a la entrada y salida, respectivamente, de la EDAR de la localidad, lo que

Figura 4. Presencia (ng L^{-1}) de fármacos en aguas superficiales. (a) Analgésicos y anti-inflamatorios no esteroides; (b) Antibióticos



[a] [296], [b] [299], [c] [300], [d] [294], [e] [301], [f] [293], [g] [251], [h] [298], [i] [295], [j] [302], [k] [208], [l] [303], [m] [273], [n] [304], [o] [119].

supuso una atenuación del 77% durante el tratamiento en la EDAR. Pero finalmente, no fue detectado en las muestras correspondientes a el río Rio Grande [119].

La contaminación por fármacos en GW, está mucho menos estudiada. No obstante, la presente revisión bibliográfica incluyó algunos estudios como el llevado a cabo por [305] que valoraba el impacto de una antigua industria farmacéutica cerca de Berlin sobre las aguas subterráneas de la zona, y donde observaron altas concentraciones de fenazona y propifenazona (hasta 4 µg L-1), y un TP del DMAA, en concreto, el AMDOPH (hasta 1 µg L^{-1}). Otro estudios incluidos en la revisión informaban de otros fármacos como el paracetamol a 211 ng L^{-1} en pozos franceses [306], y del ácido clofíbrico en aguas subterráneas de Alemania con niveles de hasta 125 ng L^{-1} [301]. También, la presencia de ibuprofeno, ácido salicílico, gemfibrocilo, naproxeno, indometacina, y bezafibro fueron detectados en efluentes de fosas sépticas localizados en Ontario, Canadá, en concentraciones que alcanzaban los 2150, 480, 430, 300, 4 and 12 ng L^{-1}, respectivamente, algunos de los cuales se seguían detectando a unos pocos µg L^{-1} a profundidades entre 10 y 20 m, flujo abajo [307]. En general, la carbamacepina es de los fármacos que más interés suscita en los estudios de aguas residuales, debido a que, por su persistencia, puede ser usada como indicador para observar zonas de influencia antropogénica a la contaminación de acuíferos, sedimentos y suelos, entre otros compartimentos medioambentales [308, 309]. Así por ejemplo, la carbamacepina fue detectada en transectos de sistemas de filtración en orilla, a concentraciones de hasta 465 ng L^{-1} entre 5 y 10 m de profundidad [301].

Los metabolitos, a menudo se detectan en los influentes y efluentes de las EDARs, pero no sistemáticamente en las aguas naturales. Así, por ejemplo, un estudio de concentraciones de 5 TPs de la carbamacepina (10,11- dihidro-10,11-epoxycarbamacepina, 10,11-dihidro-10,11-dihidroxi-carbamacepina, 2-hidroxicarbamacepina, 3- hidroxicarbamacepina, and 10,11-dihidro-10-hidroxicarbamacepina) en efluentes de la EDAR de Peterbourough, en Canadá, mostró concentraciones de, respectivamente, 426, 52, 1325, 132, 101 y, 9 ng L^{-1}, aunque solo la carbamacepina y el 10,11-dihydro-10,11-dihydroxycarbamazepine fueron detectados en las SW receptoras a 0.7 and 2.2 ng L^{-1}, respectivamente [44]. Estos resultados no son coherentes con la baja tasa de excreción de la carbamacepina [36]. Y es que, a pesar de su baja excreción, varios estudios de la carbamacepina, muestran concentraciones que alcanzan los ug/L en SW [296]. De ahí se extrae que la persistencia de la carbamacepina puede resultar de roturas a partir de sus metabolitos conjugados por parte de micro-organismos durante el tratamiento en las EDARs [38] o en el medioambiente [310].

Otra interesante revisión bibliográfica de presencia de fármacos, fue la llevada a cabo por [311], en la que se hizo un análisis de niveles de fármacos en efluentes de EDAR y aguas continentales dulces durante los años 2006-2009, de manera diferenciada para tres de las zonas más desarrolladas del planeta: Norte América, Asia-Australia y Europa. La información extraída para una selección de fármacos pertenecientes a varias familias está extraída en la Tabla 6.

Tabla 6. Datos de presencia y concentración de una selección de fármacos durante los años 2006 y 2009 en efluentes de EDAR y aguas dulces superficiales de Norte-América, Asia/Australia y Europa.

Compuestos	Intervalo de concentraciones (ng L^{-1})					
	Norte América		Asia/ Australia		Europa	
	Efluente de EDAR	Agua dulce	Efluente de EDAR	Agua dulce	Efluente de EDAR	Agua dulce
Antibióticos						
Trimetoprim	<0.5-7900	2-212	58-321	4-150	99-1264	0-78.2
Ciprofloxacino	110-1100	-	42-720	23-1300	40-3353	-
Sulfametoxazol	5-2800	7-211	3.8-1400	1.7-2000	91-794	<0.5-4
Analgésicos y anti-inflamatorios						
Naproxeno	<1-5100	0-135.2	128-548	11-181	450-1840	<0.3-146
Ibuprofeno	220-3600	0-34.0	65-1758	28-360	134-7100	14-44
Ketoprofeno	12-110	-	-	<0.4-79.6	225-954	<0.5-14
Diclofenaco	<0.5-177.1	11-82	8.8-127	1.1-6.8	460-3300	21-41
Ácido salicílico	47.2-180	70-121	9-2098	-	40-190	<0.3-302
Ácido mefenámico	-	-	4.45-396	<0.1-65.1	1-554	<0.3-169
Acetaminofén	-	24.7-65.2	1.8-19	4.1-73	59-220	12-777
Antiepilépticos						
Carbamacepina	111.2-187	2.7-113.7	152-226	25-34.7	130-290	9-157
Beta-bloqueantes						
Propranolol	-	-	50	-	30-44	20
Atenolol	879	-	-	-	1720	314
Reguladores de lípidos						
Ácido clofíbrico	n.d. - 33	3.2-26.7	154	22-248	27-120	1-14
Gemfibrocilo	9-300	5.4-16	3.9-17	1.8-9.1	2-28.571	-
Bezafibrato	n.d.-260	-	-	-	233-340	16-363

Artículos usados: [269, 276, 312-314], [36, 56, 251, 260, 262, 277, 279, 304, 315-324]
n.d.: no detectado; -: N/A

Esta tabla hace de nuevo evidente la atenuación esperada de las concentraciones en las masas de agua dulce con respecto a los efluentes de las EDARs, debido a atenuación natural por dispersión, biotransformación, fotólisis, adsorción, volatilización o combinación de estos [325]. Sin embargo no se pueden extraer conclusiones claras acerca de la distribución geográfica, y es que los intervalos de presencia observados son muy amplios. Pero en la mayoría de los casos, los fármacos más altamente presentes coinciden en todas las zonas, con excepciones como el ketoprofen o el diclofenaco, mucho más concentrados en Europa que en Norte-América o Asia/Australia, especialmente en los efluentes de EDAR, lo que puede deberse a diferencias en el uso regional de la droga y/o la eficacia de los tratamientos de las EDARs.

Recientemente, Jiang et. al [326] han publicado una revisión bibliográfica que incluye 14 países a nivel mundial, con la que han observado que más de 80 tipos de fármacos han sido detectados en aguas residuales tratada y sin tratar, y entre ellas, las más comúnmente encontradas son el ibuprofen, el naproxeno, la eritromicina y la roxitromicina, lo que refleja la disribución y el gran uso de estos productos farmacéuticos. De nuevo, apuntan a los anti-inflamatorios y antibióticos, como las drogas más abundantes en el medio ambiente acuático, con concentraciones de varias docenas de μg L^{-1} en agua residual sin tratar y tratada en el Reino Unido, Canadá y Japón. Y en consecuencia, las concentraciones de ibuprofeno, eritromician y naproxeno en aguas superficiales alcanzaron niveles de μg L-1 en estos países. Observaron sin embargo, que las concentraciones de estos compuestos en otros países era más baja, lo que lo atribuyeron a diferencias en la cantidad de uso de estas drogas entre países.

3.2 Presentación de los resultados

Como se ha comentado durante el capítulo 1 de la presente memoria, la presencia de elementos exógenos con toxicidad potencial o de facto como los fármacos y sus derivados, puede suponer un problema medioambiental. Para conocer el alcance de éste, tomar medidas y hacer un seguimiento de las mismas, es necesario contar con herramientas como los métodos analíticos descritos y discutidos en el capítulo 2. En el presente capítulo se presentan los resultados obtenidos en tres análisis del alcance (Estudios 2, 3 y 4) y uno de seguimiento (Estudio 1), que constituyen estudios de alto interés medioambiental e innovación, que arrojan luz acerca de la situación actual de los fármacos y sus derivados en las aguas medioambientales, mediante la aplicación de los métodos desarrollados en el capítulo 2:

***Estudio 1*:**

Publicación científica 5: "Assessing the effects of tertiary treated wastewater reuse on the presence emerging contaminants in a Mediterranean river (Llobregat,NE Spain)" por Rebeca

López-Serna, Cristina Postigo, Juan Blanco, Sandra Pérez, Antoni Ginebreda, Miren López de Alda, Mira Petrović, Antoni Munné, Damià Barceló en “Environmental Science and Pollution Research”

***Estudio 2*:**

Publicación científica 6: “Ocurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain)” por Rebeca López-Serna, Mira Petrović, Damià Barceló en “Science of the total environment”

***Estudio 3*:**

Publicación científica 7: “Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain” por Rebeca López-Serna, Anna Jurado, Enric Vázquez-Suñé, Jesus Carrera, Mira Petrović, Damià Barceló en “Environmental Pollution”

***Estudio 4*:**

Publicación científica 4: “Multi-residue enantiomeric analysis of pharmaceuticals and their active metabolites in the Guadalquivir River basin (South Spain) by chiral liquid chromatography coupled with tandem mass spectrometry” por Rebeca López-Serna, Barbara Kasprzyk-Hordern, Mira Petrović, Damià Barceló en “Analytical and bioanalytical chemistry”

Publicación científica #5

"Assessing the effects of tertiary treated wastewater reuse on the presence emerging contaminants in a Mediterranean river (Llobregat,NE Spain)"

por:

Rebeca López-Serna, Cristina Postigo, Juan Blanco, Sandra Pérez, Antoni Ginebreda, Miren López de Alda, Mira Petrović, Antoni Munné, Damià Barceló

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RESEARCH ARTICLE

Assessing the effects of tertiary treated wastewater reuse on the presence emerging contaminants in a Mediterranean river (Llobregat, NE Spain)

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Abstract
Purpose The Llobregat River, which is characterized by important fluctuations of the flow rates, receives treated waters from WWTPs. During the years 2007 and 2008, the Llobregat River basin suffered from a severe drought which affected the supply of drinking water facilities (DWF) that rely on the exploitation of the river water. The Catalan Water Agency implemented a water reuse experiment with the objective of maintaining the river flow rate at sufficiently high level so as to ensure the supply of raw water to Barcelona's major DWF.
Method A total of 103 emerging contaminants belonging to the groups of pharmaceuticals (74), illicit drugs (17) and oestrogens (12) were determined using LC-MS/MS methods in river water samples during the water reuse campaign. The effect of the reclaimed water discharge on the river water quality, in terms of contamination loads and environmental risk (based on the concentration addition mode), is discussed.
Results Fifty-eight pharmaceuticals out of 74 monitored were detected at least in one sample. In river water upstream (site R0) majority of compounds were detected at low nanograms per litre levels, while downstream of discharge of tertiary effluent only few compounds were detected at levels higher than 100 ng L^{-1} (i.e. acetaminophen, diclofenac, erythromycin, sulfamethazine), but never exceeding 500 ng L^{-1}. The total concentration of illicit drugs was found to be very low at both sampling sites (<50 ng L^{-1}). No relevant ecotoxicity risks were identified, except for pharmaceuticals vs. algae.
Conclusions In general, the discharge of reclaimed water in the river influenced perceptibly in terms of concentrations, mass loads and environmental risk, especially for pharmaceuticals and in less extent for illicit drugs. Nevertheless, it was not very significant in any case.

Keywords Emerging contaminants · Pharmaceutical · Illicit drugs · Oestrogens · LC-MS · Wastewater treatment · Reclaimed water · Llobregat River · Mass balances · Risk assessment · Hazard quotients · Oestrogenic equivalents

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1 Introduction

Studies on water reuse are gaining relevance due to the growing water demand in agriculture and household. (Markus and Deshmukh 2010; Azar et al. 2010; Muñoz et al. 2009a; Aggeli et al. 2009; Xu et al. 2009; Wintgens et al. 2008; Ghermandi et al. 2007; Dombeck and Borg 2005; Exall 2004). This is particularly important in areas which suffer from drought episodes and therefore their basins present important fluctuations in the flow rates like Llobregat River (NE Spain). At the same time, the catchment area is subject to heavy pressures of contamination coming from extensive urban, industrial and agricultural activities. During the period of 2007–2008, the Llobregat River basin suffered from a severe drought that caused severe problems of water scarcity. This situation seriously diminished the flow of the river and therefore indirectly threatened the supply of drinking water in the region, especially in highly populated areas like the city of Barcelona (Cazurra 2008; Mujeriego et al. 2008).

During a drinking water sources crisis, due to a severe drought that occurred in 2008, the water authority managing water resources in the area of Barcelona considered augmenting drinking water supplies with reclaimed water. To know the possible environmental, health and water quality effects of such water reuse, an experiment consisting of introducing reclaimed water in the river upstream of the potabilisation plant was performed during the autumn of 2008 and 2009. The climatic and geographic scenarios of such experiment, as well as the operational conditions, are described in detail in the Supplementary information 1. This work deals with the second campaign (November 2009), while the results of the previous one (autumn 2008) were already reported elsewhere (Köck-Schulmeyer et al. 2010). All the experiments were monitored closely by the responsible authorities in fulfilment of the existing regulations (Spanish Royal Decree 1620/2007). This refers basically to conventional physical–chemical and microbiological contaminants, whereas the so-called emerging contaminants are not yet covered therein. The aim of the present work was to monitor three classes of emerging contaminants, namely pharmaceuticals (74), illicit drugs (17) and oestrogens (12), since many of these bioactive compounds show an incomplete removal by conventional wastewater treatment technologies (Barceló and Petrovic 2007; Batt et al. 2007; Matamoros et al. 2009; Kasprzyk-Hordern et al. 2009; Pérez and Barceló 2008). Occurrence data of these compounds at strategic sampling locations and at suitable time sites are described and compared with the work by (Köck-Schulmeyer et al. 2010). The results presented there, in that first campaign, pointed out that the effect of reclaimed water discharge was not negligible in terms of micro contaminants' presence. But still, levels were comparable or even below to those reported previously in the Llobregat River and in other Spanish rivers discharging into the Mediterranean Sea.

As far as we know, this study, along with the one presented by (Köck-Schulmeyer et al. 2010), are the first papers evaluating the effects of reclamation water to guarantee the availability of inlet water in a drinking water treatment plant, in terms of emerging contaminants occurrence.

Moreover, in order to get a proper interpretation of the findings and to assess the effect of the tertiary effluent on the receiving surface water quality from a quantitative point of view, variability of both flows and concentrations was taken into account by using load (mass flow) balances. Finally, a preliminary risk assessment for the concentrations found in the river sampling sites was carried out for the three groups of contaminants considered, through the estimation of their associated hazard quotients and oestrogenicity.

2 Materials and methods

2.1 Campaign description

2.1.1 Study site

The study site and the conditions under which the experiment of releasing reclaimed water into the Llobregat River were run, are described in detail in Supplementary information 1. The study was carried out in the last part of Llobregat River (NE Spain) between the towns of Molins de Rei (site R0) and Sant Joan Despí (site R2; Fig. 1). The latter is located ca. 8.5 km downstream at the inlet of an important drinking water treatment plant, supplying water to the city of Barcelona after appropriate potabilisation treatment. The effluent of the tertiary treatment of the WWTP El Prat de Llobregat (site RW2) was pumped upstream ca. 16.6 km and discharged into the river at ca. 0.2 km below site R0.

2.1.2 Flow measurements

Flow measurements of the Llobregat River are regularly performed by the Catalan Water Agency (ACA) in the gauge flow station located in Sant Joan Despí (site R2). Measurements are recorded every 5 min and the data aggregated at day intervals. They are accessible at the ACA website (Agència Catalana de l'Aigua-Generalitat de Catalunya; http://www.gencat.cat/aca/). Tertiary effluent flows were also available from the WWTP records. River flow at site R0 (river upstream) Q_{R0}, though not measured, was

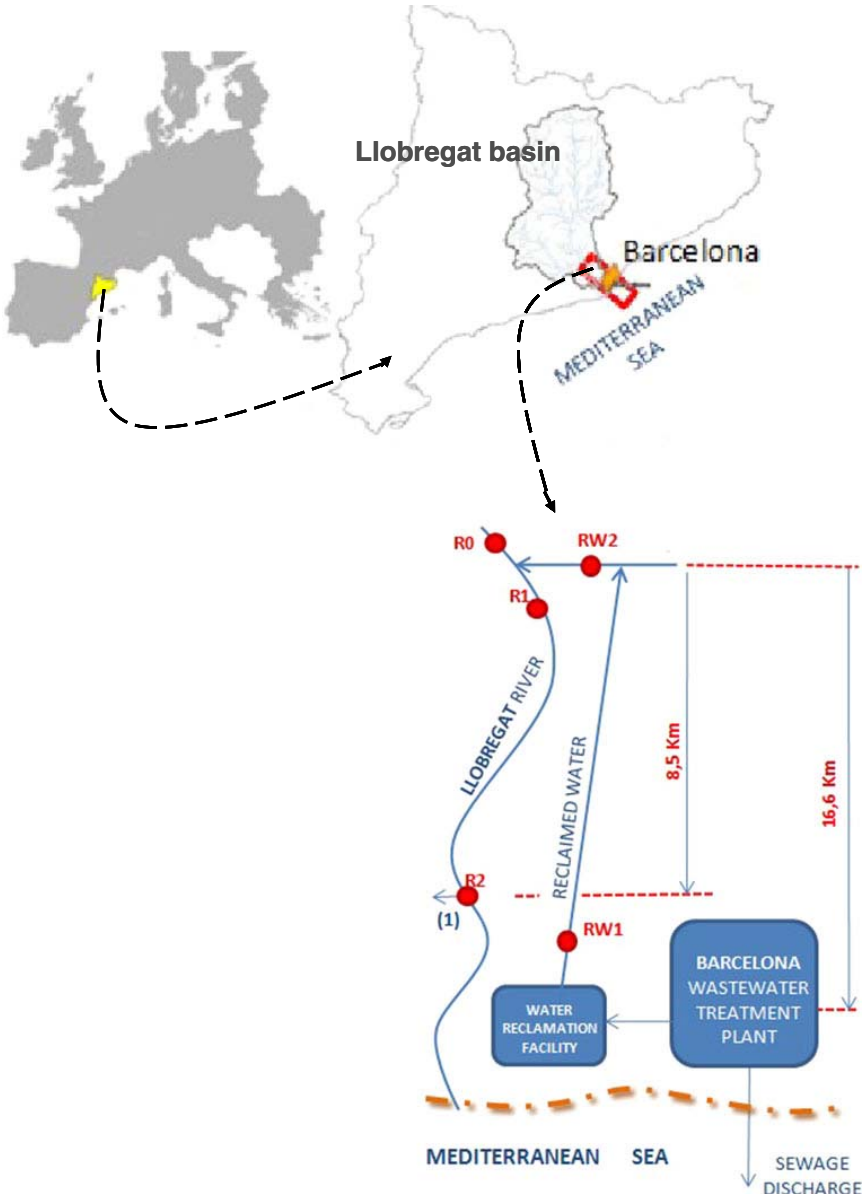


Fig. 1 Sampling points location in the lower stretch of the Llobregat River. Point *R0* Llobregat River at Molins de Rei, Point *R2* Llobregat River at Sant Joan Despí; Point *RW2* discharge of tertiary effluent from WWTP El Prat de Llobregat

assumed to be the difference between that of site R2 (river downstream) Q_{R2} and site RW2 (WWTP effluent), Q_{RW2} since the distance between both sites was not too long, and no other significant water discharges and/or water abstractions were located in that section of river. Therefore, under the foregoing assumption the flow in site R0 could be reasonably estimated by subtraction to the flow value at site R2 that of RW2.

Flow values for sites R0, R2 and RW2 corresponding to the sampling dates, either measured (R2 and RW2) or calculated (R0) are listed in Table 1.

2.2 Chemical analysis

2.2.1 Chemical reagents

Organic solvents were HPLC grade. Acetonitrile and methanol were from Riedel de Häen (Steinheim, Germany). Target compounds (Tables S1, S2 and S3—Supplementary information) were of high-purity grade (pharmaceutical >90%; drugs of abuse and oestrogens >96). In Supplementary information 2, further information about the suppliers of the standards is presented.

Table 1 Flow data (in cubic metres per second) for the different sites (R0: River Llobregat at Molins de Rei; R2: Llobregat River at Sant Joan Despí; RW2: WWTP tertiary effluent discharged)

Site reference Sampling date[a]	River upstream (R0) $Q_{R0}=Q_{R2}-Q_{RW2}$	River downstream (R2) Q_{R2}	WWTP effluent (RW2) Q_{RW2}	Dilution ratio Q_{RW2}/Q_{R2}
2–3/11/2009	3.71	4.77	1.06	0.22
5–6/11/2009	1.33	2.01	0.68	0.34
9–10/11/2009	1.7	2.47	0.77	0.31
12–13/11/2009	1.77	2.44	0.67	0.27
16–17/11/2009	1.74	2.62	0.88	0.34
19–20/11/2009	0.38	1.52	1.14	0.75

[a] Sampling was carried out taking into consideration the lag time of the river water circulation between R0 and R2

2.2.2 Sampling and sample preparation

The sampling campaign was carried out in November 2009. Water samples (5 L) corresponding to sites R0 and RW2 (see Fig. 1) were collected in amber glass bottles, on six different days. Taking into account the flow rates and the experience obtained during the 2008 campaign, grab samples were taken at site R2 on the following day. The time elapsed between the two sampling events was deemed sufficient to allow the water sampled at R0 and RW2 to reach site R2. During the transportation of the samples to the lab, they were kept in dark, cooled inside chambers with ice and stored at −20°C until the analysis. Upon reception in the lab, samples were immediately filtered through 1-μm glass fibre filters, followed by 0.45-μm nylon membrane filters (Whatman International Ltd, Maidstone, England), and stored in the dark at −20°C (in freezers) until analysis.

2.2.3 Analysis method

Sample preparation and preconcentration was performed according to previously reported validated methods by (López-Serna et al. 2010) for the analysis of the 74 pharmaceuticals; by (Postigo et al. 2008) for the 17 illicit drugs and by (Rodriguez-Mozaz et al. 2005) for the analysis of the 12 oestrogens. All the methods consist of online SPE-LC-MS/MS. To control the performance of the analytical methods for all the compounds, samples were spiked with specific isotopically labelled surrogate standards prior to extraction as published previously. The methodology for the determination of oestrogens was modified in that the number of isotopically labelled surrogates was increased to six. Limits of detection and quantification for all target compounds are in Tables S4–S6 (Supplementary information).

2.3 Load contributions and mass balances

For every compound *i*, and site of sampling *j* the load (expressed as mass flow in appropriate mass/time units) was computed as the product of concentration c_{ij} per flow Q_j: Hence, assuming a conservative behaviour for the contaminants (see Section 3.2), for every compound *i* the following mass flow balance equation holds:

$$\mathbf{Q}_{R2} \cdot \mathbf{c}_{R2i} = \mathbf{Q}_{R0} \cdot \mathbf{c}_{R0i} + \mathbf{Q}_{RW2} \cdot \mathbf{c}_{RW2i} \quad (1)$$

3 Results and discussion

3.1 Occurrence of micro contaminants

The occurrence of 103 emerging contaminants was studied in both the Llobregat River (Fig. 1, sites R0 and R2) and the effluents pumped upstream and discharged into the river from the WWTP El Prat de Llobregat (Fig. 1, site RW2) after an adequate tertiary treatment (as described in Supplementary information 3). The results are summarized in (Tables S1, S2 and S3 supplementary information). Generally, pharmaceuticals were found to be present at higher concentrations than illicit drugs and oestrogens in both river water (Fig. 2a–c) and treated wastewater (Fig. 3a–c).

As far as pharmaceuticals are concerned (see Fig. 2a and Table S1 Supplementary information), 58 compounds out of 74 monitored were detected at least in one sample. In river water upstream of discharge of tertiary effluent (site R0) 39 compounds were detected at low nanograms per litre levels, while three analgesics and anti-inflammatory drugs (acetaminophen, ibuprofen and salicylic acid) were the only ones found at levels higher than 100 ng L^{-1}, but rarely exceeding 300 ng L^{-1}. Other frequently detected compounds comprise anti-inflammatories diclofenac and naproxen, β-blockers atenolol and metoprolol, antibiotics ofloxacin, erythromycin, clarithromycin and sulfamethoxazole and psychiatric drugs carbamazepine and lorazepam. However, in all cases concentrations detected were lower than 100 ng L^{-1}.

In treated wastewater (site RW2) levels were higher, but still at <1,000 ng L^{-1} level for the majority of compounds (Fig. 3a). The exception were three antibiotics (sulfamethazine, azithromycin and erythromycin) and the diuretic furosemide that were detected at low micrograms per litre (max concentration found was 3.08 μg L^{-1} of

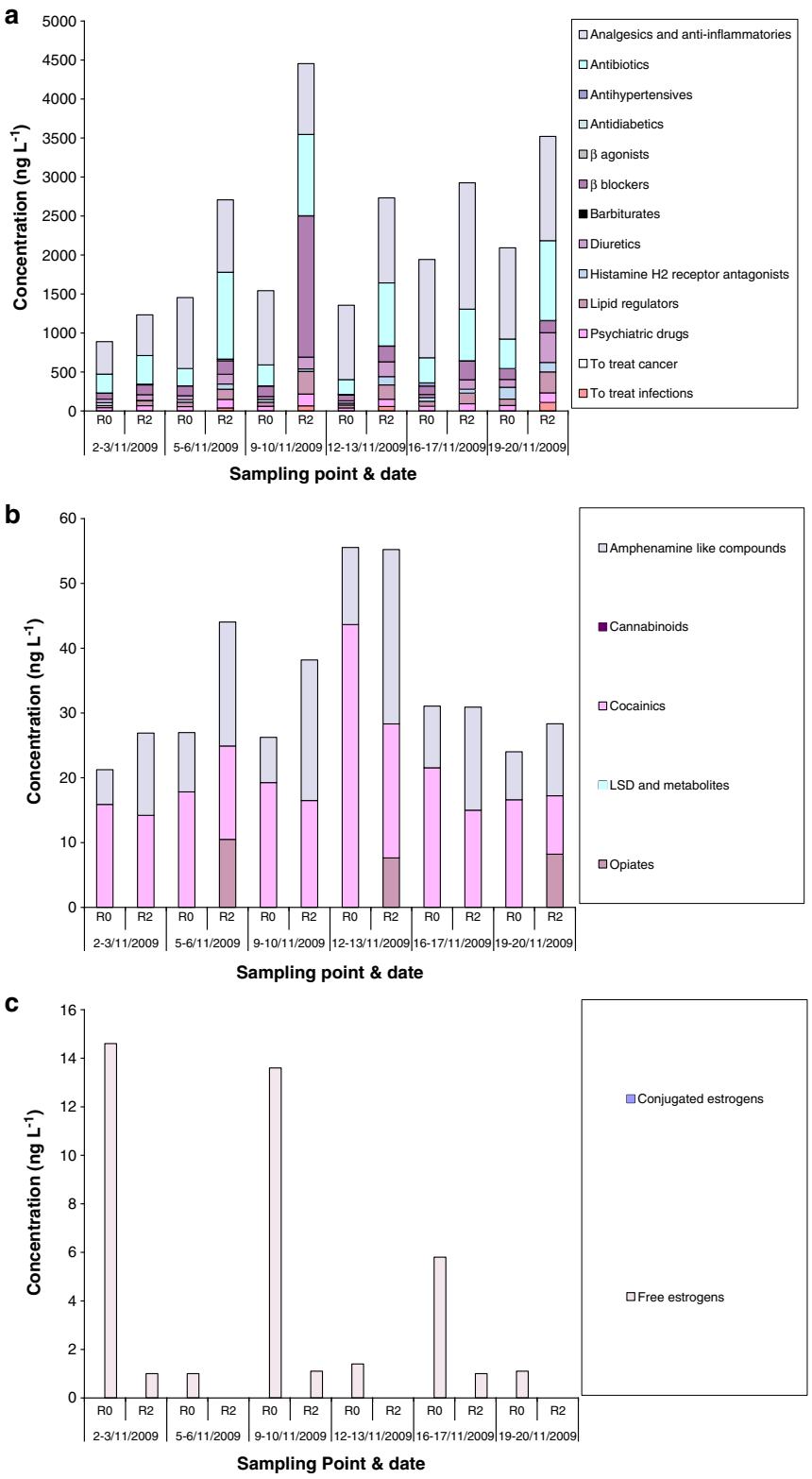


Fig. 2 Concentrations (in nanograms per litre) per families of compounds in river upstream (point *R0*) and downstream (point *R2*) to discharge the point. **a** Pharmaceuticals; **b** illicit drugs and **c** oestrogens

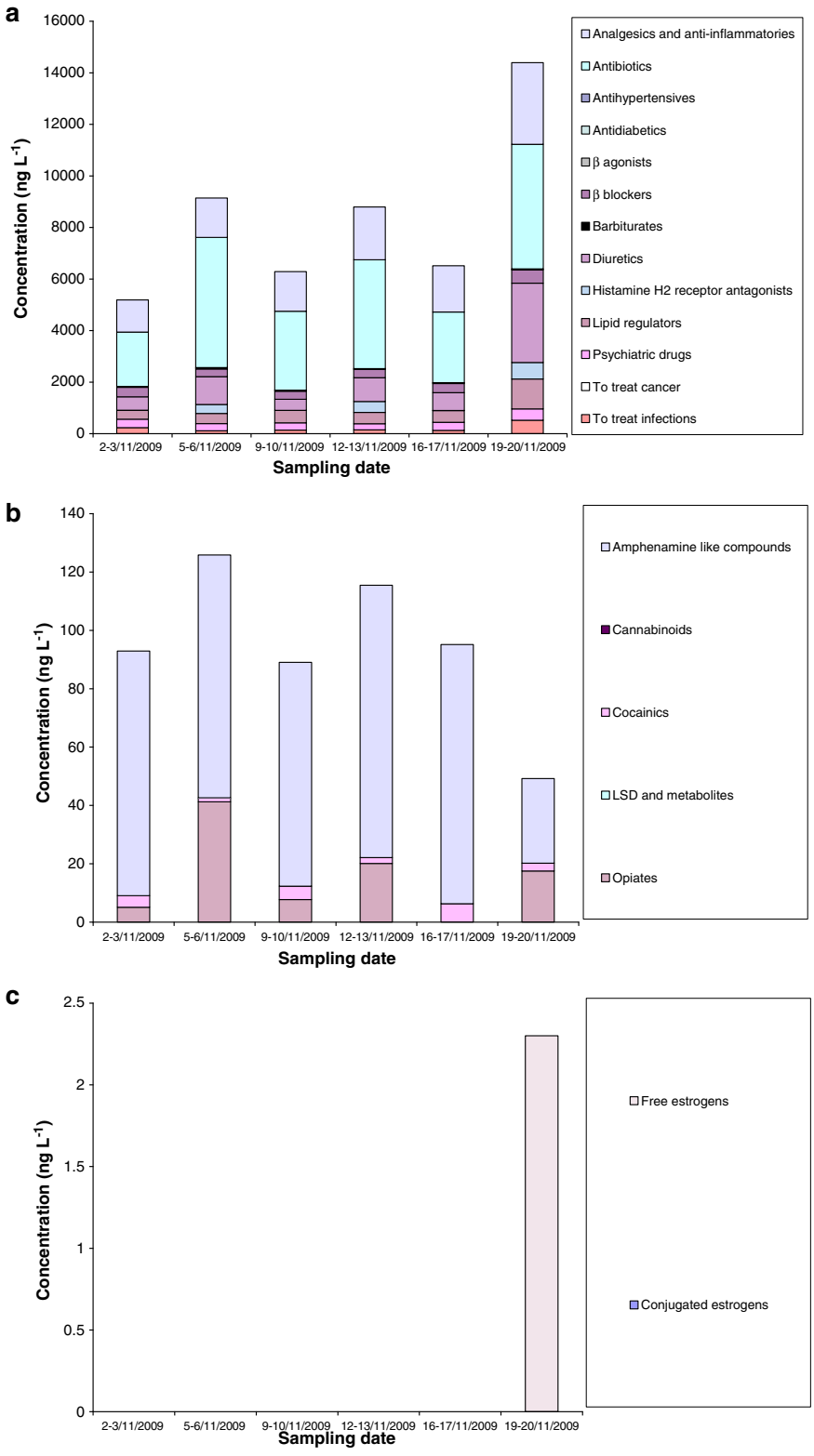


Fig. 3 Concentrations (in nanograms per litre) per families of compounds in tertiary WWTP effluent (point RW2). **a** Pharmaceuticals; **b** illicit drugs and **c** oestrogens

furosemide in one sample) and several other compounds were detected occasionally at levels between 100 and 900 ng L^{-1} (codeine, salicylic acid, atenolol, enrofloxacine, ofloxacin, bezafibrate, fenofibrate and metronidazole).

Observing concentrations in river water (site R0 upstream of discharge and site R2 downstream of discharge of tertiary effluent; Fig. 2a), it is visible that the discharge of the tertiary effluent affects the levels of pharmaceuticals, but the increase in concentrations is not significant. At site R2 only few compounds were detected at levels higher than 100 ng L^{-1} (i.e. acetaminophen, diclofenac, erythromycin and sulfamethazine), but only the salicylic acid exceeds 500 ng L^{-1}.

In general, the compounds found at higher concentrations and the proportion among them coincided with the ones in (Köck-Schulmeyer et al. 2010), but levels were higher in the present study. In fact, the total concentration of pharmaceuticals in the river upstream (RO) and downstream (R2) the discharge point, was two- and fourfold higher, respectively. In any case, levels were comparable or even below to those reported previously for the Llobregat River and in other Spanish rivers discharging into the Mediterranean Sea (Farré et al. 2001; Kuster et al. 2008; Ginebreda et al. 2010; Muñoz et al. 2009b).

Concerning illicit drugs and their metabolites (see Fig. 2b and Table S2, Supplementary information), 10 out of the 17 investigated compounds were not detected in any of the investigated samples. The whole families of cannabinoids and LSD compounds were absent in water phase, due to very low excretion rate and lypophilicity in case of cannabinoids and due to lower relative consumption (low individual dosage) in case of LSD. This fact was also observed in the study realized in 2008 (Köck-Schulmeyer et al. 2010). On the other hand, cocaine and benzoylecgonine, and the amphetamine-like compound ephedrine were present in all water samples analysed, ephedrine being the one that was found at the highest concentration, (79.5 ng L^{-1} in the tertiary effluent). It is interesting to mention that cocaine and its major metabolite benzoylecgonine presented higher average concentrations in river upstream (site R0) than in discharged treated water (site RW2). The Llobregat River is subjected to a heavy anthropogenic pressure, receiving extensive urban and industrial waste water discharges. It receives the effluent discharges of more than 55 WWTPs, and both sampling sites (R0 and R2) are situated in the lower part of the river, which explains why levels of some contaminants are higher in river water than in effluents of an efficient WWTP such is the case of WWTP El Prat. Regarding opiates, morphine was the only one detected, with a maximum concentration of 41.2 ng L^{-1} in treated effluent.

The impact of the tertiary discharge into the river was even lower than the one observed for pharmaceuticals (see Figs. 2b and 3b). Only small, not significant, increase in concentrations is observed at sampling site R2 for some compounds (ephedrine, ecstasy and metamphetamine), while for others the levels were lower. Generally, the total concentration of illicit drugs was found to be very low at both sampling sites (<50 ng L^{-1}). In fact, in contrast to the pharmaceuticals, the levels found in the present study, were lower than the ones presented by (Köck-Schulmeyer et al. 2010), where, nevertheless, they did not surpass 100 ng L^{-1} in total. Also in terms of presence, the number of illicit drugs detected was lower in the present study than in previous campaign.

Regarding the oestrogens (see Fig. 2c and Table S3, Supplementary information), the positive findings correspond to oestrone, that was detected in both river water and treated wastewater (see Figs. 2c and 3c) at levels never exceeding 3 ng L^{-1}, and diethylstilbestrol that was found at sampling site R0 (river upstream of discharge of tertiary effluent). Occurrence of diethylstilbestrol is especially interesting since it is a banned substance, and its presence is probably due to its fraudulent use as a growth promotant in farms. It was found only at sampling site R0 at average concentration of 4.5 ng L^{-1} and maximum level of 12.2 ng L^{-1}. These two compounds were also quantified in the study performed by (Köck-Schulmeyer et al. 2010) at concentrations tenfold higher for the oestrone and at comparable levels for the diethylstilbestrol. Also, in the present study, the number of oestrogens detected were lower, i.e. only two versus eight. The presence of those two oestrogens was also reported in the Llobregat tributary, Cardener River, by (Petrovic et al. 2002).

3.2 Load contributions and mass balances

To have a better insight on the relative influence of both river upstream and effluent discharge to the overall amount of pollutants found downstream in the river, direct comparison of concentrations is not adequate, since they do not take into account the respective flow. Instead, loads (expressed as mass flow in mass/time units) may provide a more convenient way to quantify and compare the relative contribution of each stream (river and effluent). This is especially relevant, since one of the objectives of the reuse experiment was to study the effect of the dilution ratio of the reclaimed water with respect to the river in the overall pollution. Therefore, loads for sites R0, R2 and RW2 were calculated as the product of concentrations per flows for the three groups of pollutants studied (Section 2.3). Compounds whose levels were below the limit of detection and/or quantification were set to zero.

It can be reasonably assumed that the load found downstream (site R2) was only determined by the mixture of the load coming from river upstream (site R0) with that brought by the effluent discharged (site RW2). Since sampling site R0 and the discharge site RW2 were at very close distance (ca. 0.2 km) other influences in such a short stretch may be considered negligible, thus allowing comparison with their relative load contributions without much error. The results obtained at the different sampling dates and locations, expressed as percentages, are depicted in Fig. 4a–c for the three classes of compounds studied. The effluent contribution (RW2) seems to predominate over that of the river upstream (R0) for both pharmaceuticals and illicit drugs, accounting for ca. 40–95% and ca. 50–90% of the total load, respectively. This contribution was slightly higher than the one reported in 2008 (Köck-Schulmeyer et al. 2010), probably due to lower effluent dilution ratios. As expected, for both classes of compounds, the highest effluent contributions were observed with the lowest effluent dilution (highest ratio Q_{RW2}:Q_{R2}=0.75), which corresponds to the last sampling date (see Table 1). That can also explain that the contamination for the oestrogens that day comes mainly from RW2 in contrast with the rest of the sampling days, when the main contribution was coming from the river. This is in sharp contrast with the preceding campaign (2008) (Köck-Schulmeyer et al. 2010), in which oestrogens were exclusively traced to the effluent. Nevertheless, due to these analytes are scarcely detected, and when quantified, they are found at very low concentrations (ca. 1–10 ng L^{-1}), trustworthy conclusions cannot be drawn.

Loads (mass flows) also provide a straightforward way to compare in bulk the weight of the different pollutant families studied in quantitative terms. The results corresponding to site R2 (river downstream), along the whole sampling period, are summarized in Fig. 5. From them, the following rank order was observed (concentration ranges are given in parentheses):

Pharmaceuticals (10^7–10^6 ng/s) >> illicit drugs (10^5–10^4 ng/s) > oestrogens (10^4–b.l.d. ng/s)
(b.l.d: below the limit of detection)

This order shows a clear predominance of pharmaceuticals over the other classes, followed by illicit drugs and oestrogens, which is in full agreement with the rank found in the study of 2008 (Köck-Schulmeyer et al. 2010). To our knowledge, apart from that latter work and the present one, no additional data regarding the loads of these emerging contaminants is currently available in the literature.

Mass balances compliance was further checked upon the assumption of a conservative behaviour for the contaminants through the calculation of concentrations in site R2. This is a reasonable assumption given the short distance travelled. Therefore, since both flow and concentrations at site R2 (Q_{R2} and c_{R2i}) are already available from direct

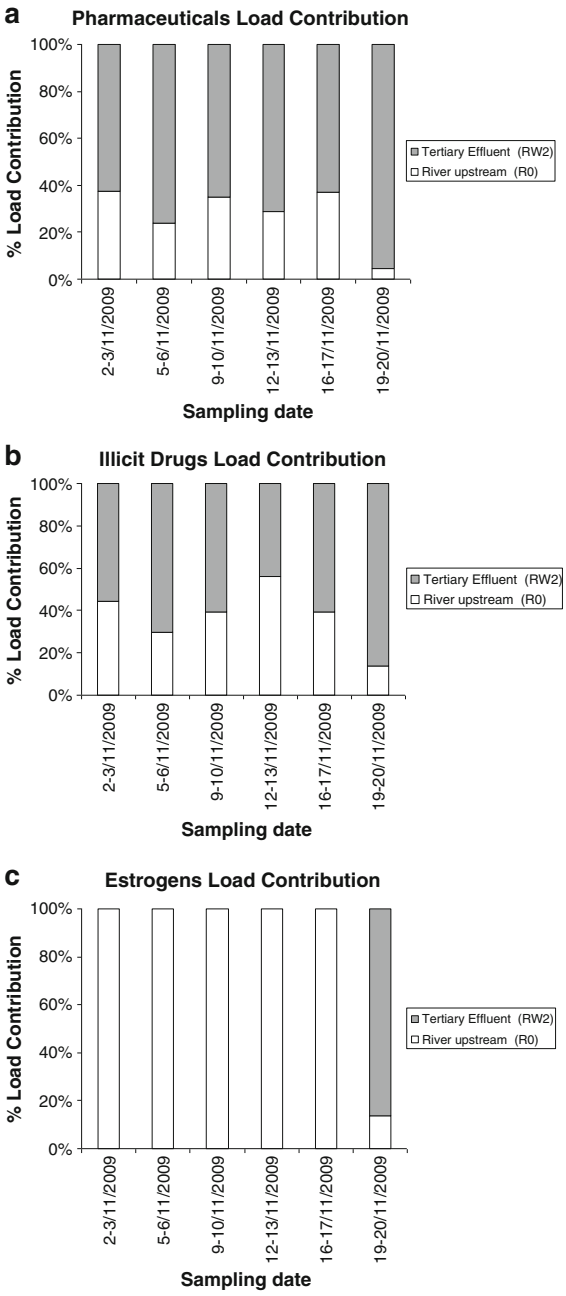


Fig. 4 Mass load relative contributions (%) of river upstream and tertiary effluent in point R2 at the different sampling dates. **a** Pharmaceuticals; **b** illicit drugs and **c** oestrogens

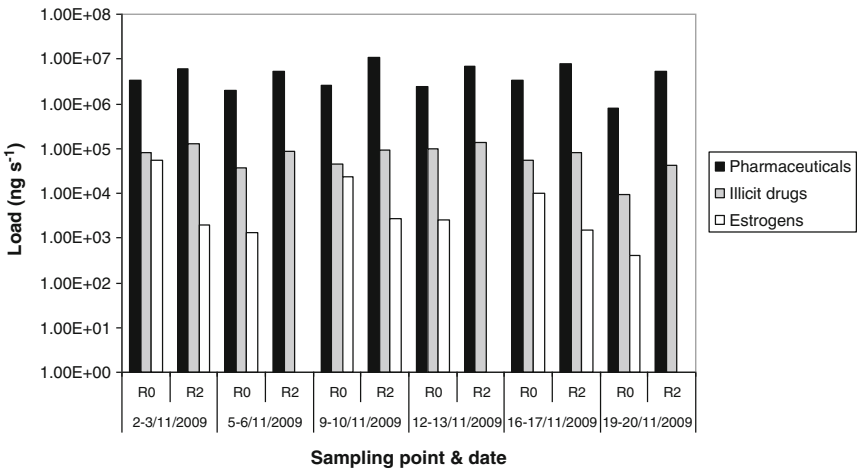


Fig. 5 Comparison of mass loads (nanograms per second) per compound classes in points R0 (river upstream) and R2 (river downstream) at the different sampling dates

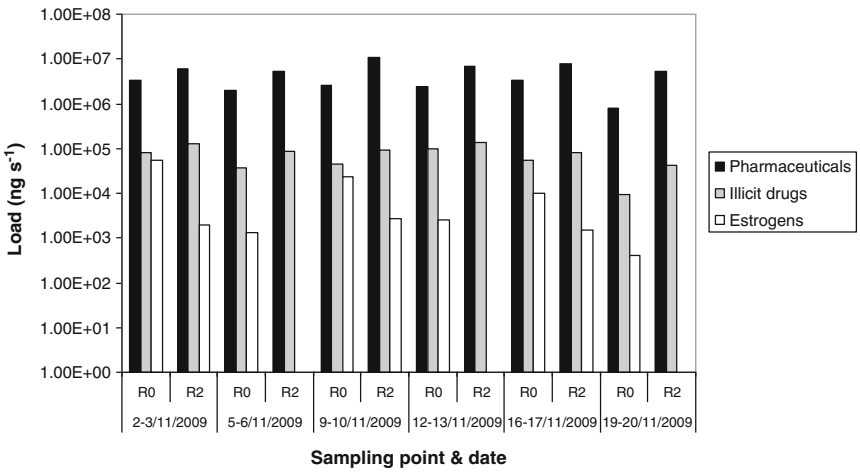
measurements, the predicted value of c_{R2i}(calc), which was straightforward calculated from the foregoing mass balance equation (Section 2.3, Eq. 1) may be compared to the experimental c_{R2i}(exp). The suitability of these assumptions was thus experimentally tested. Figure 6a and b show the graphs obtained representing experimental vs. calculated concentrations according to the outlined procedure for pharmaceuticals and illicit drugs, for the different sampling dates. Even though there is an acceptable agreement between experimental and calculated values, the later tend to be overestimated, especially for the pharmaceuticals (the linear regression coefficients together with the 99% confidence intervals are shown on the charts). In fact, 76% of the concentration pairs are in this situation both for pharmaceuticals and illicit drugs. This could be due to several reasons, all of them attributable to deviations from the balance Eq. 1 related to the non-fulfilment of the implicit assumption of conservative behaviour along the river segment considered either of flows or concentrations. The following possibilities are suggested among others as plausible causes: (1) disappearance of the compound by some kind of depletion mechanism occurring in the river, such as physical–chemical (e.g. photo degradation, adsorption onto sediments, …) or biological degradation, and (2) by the possible existence of some flow alteration neglected in the balance equation that might contribute to alter the dilution factor (Q_{R2}), such as depletion caused by infiltration or, in the opposite sense, the occurrence of some temporal minor water stream reaching the Llobregat River between sites R0 and R2. Whereas the second factor would be expected to equally affect all compounds, the former appears more likely since it was compound specific. However, no clear relations can be established between the data of physico-chemical or biological degradation found in the literature, and the overestimated results observed. Thus, the epileptic drug, carbamazepine, is known to be highly resistant to depletion, either by soil adsorption (Nakada et al. 2008; Scheyt et al. 2006; Chefetz et al. 2008), photo degradation (Chiron et al. 2006; Andreozzi et al. 2002) or biologically (Nakada et al. 2006), but in the present study, it presents overestimation in 5 out of 6 days of sampling.

In any case, the differences between calculated and experimental concentrations were in general very narrow, with relative standard deviations inferior to 40% in most of cases, e.g. for carbamazepine, the average experimental concentration was 58 ng L^{-1} versus an estimated one of 79 ng L^{-1}.

In summary, the mass-flow balance equation may acceptably serve to predict downstream concentrations of pollutants if flows and concentrations corresponding to both river and effluent discharges occurring upstream are known, and the distance travelled by water is moderate.

3.3 Ecotoxicological and oestrogenic assessment

Adverse ecotoxicological effects caused by pollutants on aquatic organisms are the main problem of their presence in the environment. The so-called hazard quotient (HQ hereafter) is a suitable tool to estimate the ecological risk attributable to each pollutant (Kim et al. 2007). The Water Framework Directive (Sanderson et al. 2003), stated the convenience of assessing taxa belonging to three different trophic levels. Commonly, algae, daphnids and fish are used as reference organisms. Whereas EMEA guideline proposes the calculation of this quotient as the ratio between Predicted Environmental Concentrations (PEC) and Predicted No-Effect Concentration (PNEC) (Grung et al. 2008); (Huschek et al. 2004), other authors prefer using Measured Environmental Concentrations (MEC) instead of PEC (Santos et al. 2010). Hazard quotients defined as HQ = MEC/PNEC are determined for every compound present on



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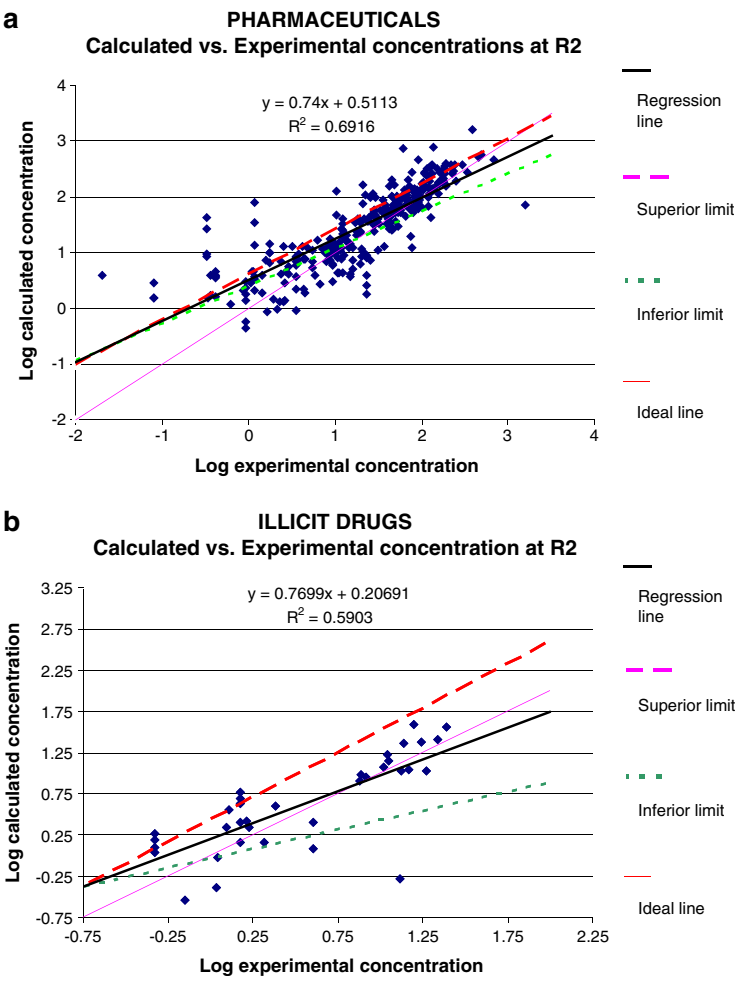

Fig. 6 Calculated vs. experimental concentrations in point R2 for **a** pharmaceuticals and **b** illicit drugs

the scenario under study. However, since PNEC data are not always available in the literature, acute toxicity values are commonly utilized. Thus, PNECs are typically calculated from EC50 values corrected by a safety factor of 1,000 as it is recommended by the Water Framework Directive (Directive 2000/60/EC). The so calculated HQ are thus equivalent to the often used toxic units (TU) (Sprague and Ramsay 1965), differing only on the correcting safety factor. Irrespectively whether HQ or TU are used, they can be aggregated by simple addition on an overall hazard quotient (HQ = ∑HQi) under the assumption of the concentration addition (CA) mode of action (Bliss 1939). CA is based on the assumption of a similar mode of action for all the substances present in the mixture. This implies that all components contribute to the final effect even at low concentrations or said in other words that concentrations can be added. Based on solid experimental support there is a wide consensus on accepting CA as a general purpose model of choice for a first approximation to mixture toxicity estimation (Backhaus 2011). Nevertheless, it must be reminded that synergic effects among compounds can take place (Santos et al. 2010), so the sum of single hazard quotients must be interpreted as a first rough approach. Upon that basis, it is usually accepted that if HQ is equal or higher than 1 potential impairment of the aquatic ecosystem is likely to occur. Ecological risks caused by micro pollutants are expected to be related with chronic toxicity. Hence, in order to take into account that chronic toxicity might occur at lower concentrations than the acute one, that safety factor of 1,000 was applied in the calculation of those PNEC. In the present work, hazard quotients have been calculated individually for the minimum and the maximum concentration found of every pharmaceutical and illicit drug compound, in river sites R0 and R2. Afterwards, a total HQ was estimated, by adding all those individual values for both groups of micro contaminants. Experimen-

tal EC_{50} values extracted from the literature were preferred, but when they were not available, EC_{50} were estimated using USEPA's ECOSAR (Ecological Structure Activity Relationships) model (ECOSAR v1.00). When more than one EC_{50} value was available, the lowest was taken into consideration (Grung et al. 2008). Therefore, the risk in the most extreme situation has been in any case considered. In Tables S7 and S8 (Supplementary information), the values of EC_{50} for fish, daphnid and green algae used for the calculation of PNEC are reported.

In what pharmaceuticals are concerned, only two out of the 74 compounds studied (ciprofloxacin and sulfamethoxazole) showed an individual HQ above 1, and in all cases that toxicity corresponded to algae. It was attributable to the low EC_{50} of the foresaid two compounds (ciprofloxacin 0.005 mg L^{-1} and sulfamethoxazole 0.027 mg L^{-1}; see Table S7, Supplementary information), which give rise to the corresponding HQ values as high as 15.6 in site R2 and 2.9 in R0 for ciprofloxacin, or 4.6 and 1.9 in R2 and R0 for sulfamethoxazole. For the other two reference species (daphnids and fish), individual HQs were below 1 in all sites of sampling. As regards as illicit drugs, all the individual HQs for the different compounds were far under 1. Thus, even upon aggregation of all the HQs for every compound, global HQ values obtained would be clearly under that limit for all the species assessed.

Hence, and as it can be observed in the charts shown in Fig. 7a, b, the environmental risks based on HQs in R0 and R2 are in general low for pharmaceuticals and negligible for illicit drugs. Pharmaceuticals vs. algae was the only exception for which the total HQ, considering the contribution of all the pharmaceuticals assessed altogether, resulted with maximum values of ca. 22 and 5 in R2 and R0, respectively. The overall relative order of susceptibility to pharmaceuticals was estimated to be green algae >> daphnid > fish in river both sites R0 and R2, whereas for illicit drugs (see Fig. 7b), the order observed was daphnid ≈ green algae > fish, being the differences among organisms less pronounced than for pharmaceuticals. HQ ranges for both pharmaceuticals and illicit drugs are slightly higher in site R2 than in site R0, though of the same order of magnitude.

Overall oestrogenicity was also determined in the same river sites R0 and R2 by addition of the individual oestrogenic activities of each compound. They were calculated as estradiol equivalent concentration (EEQ) multiplying the oestrogenic potency for every compound by its concentration. The oestrogenic potency was thus given as estradiol equivalency factors (EEFs), defined as EC_{50} of the compound relative to the EC_{50} of 17β-estradiol. In Table S9 (Supplementary information), the EEFs for the free oestrogens are shown. Values for conjugated ones were not found in the literature. When

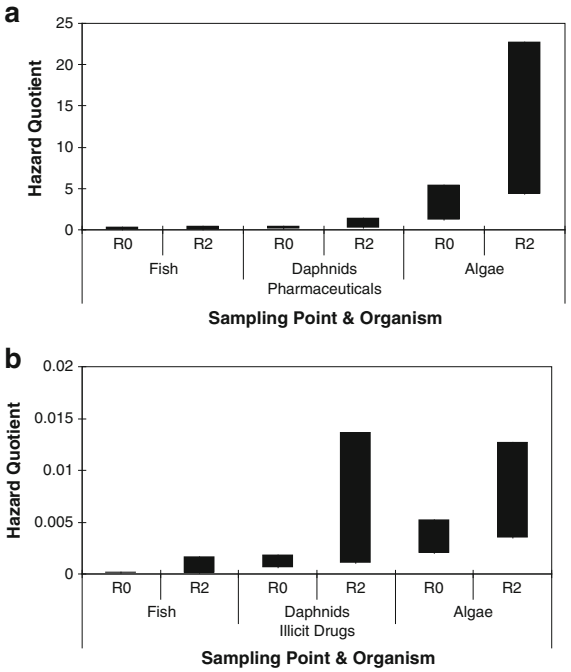

Fig. 7 Hazard quotients for pharmaceuticals at points R0 (river upstream) and R2(river downstream) for the different organisms: **a** pharmaceuticals and **b** illicit drugs

more than one value was found for the same oestrogen, the highest one was chosen in order to assess the worst situation possible. The oestrogenic activity of the analysed river waters (R0 and R2) is shown in Fig. 8 for the concentration range of two oestrogens quantified (oestrone and diethylstilbestrol). Although the levels of concentration found were quite low, they may represent some risk since oestrogenic effects in aquatic ecosystems have been reported at levels of estradiol as low as 1 ng L^{-1} (Hansen et al. 1998; Purdom et al. 1994). In the case presented, EEQs of up to 1.10 and 13.42 ng L^{-1} were determined in site R0 (river upstream) for oestrone and diethylstilbestrol, respectively. Individual values calculated for site R2 were under 1 ng L^{-1} in all cases.

4 Conclusions

In the present work, the impact of wastewater reuse on the river water quality has been examined by monitoring the occurrence of three classes of emerging contaminants, namely pharmaceuticals, illicit drugs and oestrogens. This study entails the second campaign, thus completing preliminary findings of the preceding one carried out in 2008 and allowing to compare results and to consolidate conclusions gathered from both campaigns.

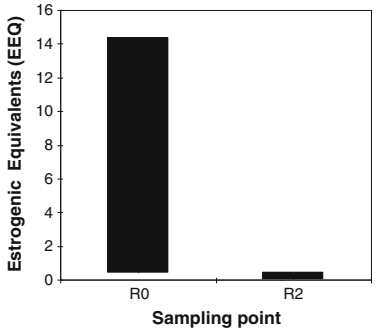

Fig. 8 Oestrogenic activity expressed as estradiol equivalent concentration (*EEQ*), associated to the oestrogens detected in R0 (river upstream) and R2 (river downstream)

As far as pharmaceuticals are concerned, comparing concentrations in river water upstream and downstream to the site of discharge of the reclaimed tertiary effluent, its effect, the increase in concentrations, though perceptible is not significant. Regarding illicit drugs and their metabolites, the impact of the discharge into the river was even lower than the one observed for pharmaceuticals. In turn, in contrast with the previous campaign, oestrogen levels were clearly higher upstream than downstream of the river.

A complementary approach aiming to distinguish the origin of the overall pollution found in the river downstream was attempted through the comparison of loads (mass flows). Whereas pharmaceuticals and illicit drugs loads were mostly allocated to the effluent, the origin of oestrogens was mainly river upstream.

Moreover, assuming mass conservation behaviour, the mass balance equation also allowed to roughly estimate the concentration of the emerging compounds downstream.

Finally, an ecotoxicity risk assessment comparing river upstream and downstream to the discharge site was performed for pharmaceuticals and illicit drugs for three reference organisms belonging to different trophic level (fish, dapnids and algae). Again, differences between river upstream and downstream to the discharge site were perceivable, but not very significant. Overall, no relevant risks were identified, with the only exception of pharmaceuticals vs. algae that were likely to occur both in river upstream and downstream. Such algae toxicity was mostly attributable to the extremely low EC_{50} reported for two compounds, i.e. ciprofloxacin and sulfamethoxazole. Oestrogenicity was similarly evaluated using estradiol equivalent factors (EEQs) for the oestrogenic compounds identified. In the present case river upstream showed higher oestrogenicity than river downstream, attributable to oestrone and diethylstilbestrol.

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Publicación científica #6

“Ocurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain)”

Por:

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Occurrence and distribution of multi-class pharmaceuticals and their active metabolites and transformation products in the Ebro River basin (NE Spain)

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ABSTRACT

The present work reports the occurrence of pharmaceuticals and their metabolites and transformation products (TPs) in the Ebro river basin (NE Spain). Twenty-four samples of water collected along the basin were analysed using a fully automated method based on on-line turbulent chromatography–liquid chromatography–tandem mass spectrometry (TFC-LC-MS/MS). In total, 17 metabolites, 7 of them with remaining pharmacologic activity, and 2 transformation products, along with 58 parent pharmaceuticals are analysed. Metabolites and TPs were found at concentrations of the same order of magnitude as their corresponding parent pharmaceuticals, with the exception of 10,11-epoxi-carbamazepine which was found at approximately 10 times higher concentration than its corresponding parent pharmaceutical carbamazepine. In general, levels of all target compounds were below 100 ng L^{-1}, with the exception of 14 compounds; among them the aforementioned 10,11-epoxicarbamazepine with a maximum concentration of more than 1600 ng L^{-1}. The analgesic propyphenazone, the psychiatric drug carbamazepine, the antibiotics clarithromycin and sulfadiazine, the cardiovascular drug propranolol, the antineoplastic tamoxifen and 1 pharmacologically active metabolite salicylic acid were found to be ubiquitous (detected in all samples). Smaller tributaries generally show higher concentrations than the main river Ebro, due to lower dilution of WWTP effluents discharged.

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1. Introduction

In Europe and USA, around 4000 different pharmaceutical active compounds are commercialised to be used as human and veterinary drugs (Howard and Muir, 2011; Mompelat et al., 2009). However, a rather small portion of them are monitored in the environment and less than 300 have been detected so far (Howard and Muir, 2011). Majority of them have been identified in surface waters (Rudén et al., 2010), as being parent active ingredients in the focus of interest, while very little attention is paid to the metabolites and transformation products.

Once in the human/animal body, the ingested pharmaceutical molecule undergoes a set of biochemical reactions. Thus, hepatic metabolism includes, the first step of oxidation/reduction and hydrolytic cleavages leading to more polar molecules than the parent compounds (Pérez and Barceló, 2007; Robinson et al., 2007). Then, a second conjugation step consists of the processes of glucuronidation, acetilation and sulphation, that allow metabolites to become hydrophilic and water soluble enough to be eliminated through urine and/or faeces (Mompelat et al., 2009). At that point, a notable specificity of metabolism is the loss of pharmaceutical activity of the native pharmaceutical compound. But this is not always fulfilled. For instance, pro-drugs, by definition, are active only after the metabolic activation by enzymatic system(s) of the parent compound to metabolite(s). Typical cases are fibrates (lipid regulators, e.g. fenofibrate or clofibrate), the cardiovascular drug enalapril (Buerge et al., 2006), etc. Therefore, pharmaceuticals, together with their metabolites, are continuously released into the environment via treated (or untreated) wastewaters, being that municipal wastewater effluent is the main route of entrance in the environment (Bound and Voulvoulis, 2005; Drewes and Shore, 2001; Heberer, 2002; Vieno et al., 2005). Manufacturing and disposal of unused and expired medicine in landfills and sewage systems (Vera-Candioti et al., 2008) are other potential sources of pharmaceuticals in the environment (see Fig. 1). Taking into account the fact that in general pharmaceuticals have a designed resistance to biodegradation, the elimination in waste water treatment plants (WWTPs) is not significant for most of the drugs (Fatta-Kassinos et al., 2011). In addition, upon partial degradation of pharmaceuticals and/or their metabolites during activated sludge treatment, transformation products (TPs) may be formed (Kern et al., 2010) and even the conjugates can be hydrolysed back to the free parent drug (Daughton, 2001; Rodriguez-Mozaz et al., 2007), which then enter the aquatic environment by the treated effluent. Once released

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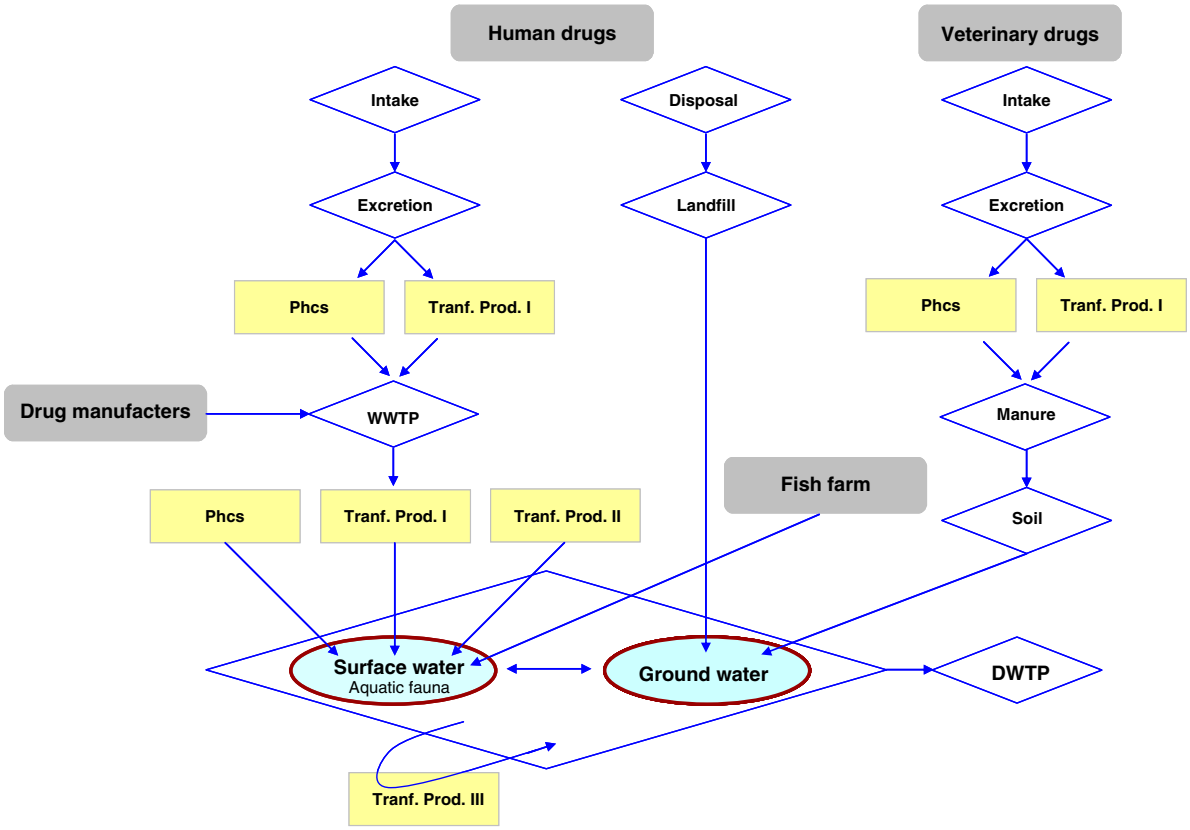

Fig. 1. Source and fate of pharmaceuticals and their transformation-products in the environment.

into the environment, pharmaceuticals can undergo transport and biotic and abiotic degradation processes (Fatta-Kassinos et al., 2011). For the same reason aforementioned, microbial degradation is considered to be a less important process in the aquatic environment. Among abiotic degradation processes (e.g. hydrolysis, photolysis, redox reactions, etc.), direct photolysis and indirect photodegradation, may be important processes for these compounds, because their structure usually contains aromatic rings, heteroatoms, and other functional groups that can either directly absorb solar radiation or react with the photogenerated transient species in natural waters. Photolytic reactions are often complex involving various competing or parallel pathways and leading to multiple products that may (i) be more toxic than the parent compound; (ii) retain the properties of the parent compound (i.e. antibiotic activity) or (iii) lose activity and/or toxicity. However, very few reports are available with regard to potential toxic effects of photo-transformation products (Fatta-Kassinos et al., 2011). Assessment of the ecotoxicity of the photoproducts of diclofenac, naproxen, and the fibrates, for instance, provides evidence that acute and chronic toxicity can be greater for the photoproducts than for the parent compounds, and genotoxic and mutagenic effects cannot be excluded (Fatta-Kassinos et al., 2011).

The possibility that pharmaceuticals and their derivates (metabolites and TP) can cause negative effects on the aquatic organisms has been widely discussed and documented in the scientific literature (Daughton and Ternes, 1999; Jjemba, 2006; Martínez Bueno et al., 2007; Petrovic et al., 2004; Petrović et al., 2003; Smital et al., 2004). Pharmaceuticals are found in the environment in the ng L^{-1}–μg L^{-1} (ppt–ppb) range. Such low concentrations of individual compounds may not pose an acute risk, however, the combined concentrations from compounds sharing a common mechanism of action could be substantial. Moreover, it cannot be excluded that pharmaceuticals and active metabolites act through additional unknown modes of toxic action on non-target organisms (Escher et al., 2005). Exposure in the aquatic environment is of particular concern, since aquatic organisms are subjected to continual unabated lifecycle exposures. In addition, the polar and non-volatile nature of most pharmaceuticals prevents their escape from the aquatic realm (Vera-Candioti et al., 2008). The concern also comes over the quality and safety of drinking water since environmental waters are often used as a source and several studies reported the occurrence of pharmaceuticals in drinking water (Trenholm et al., 2009).

In the present work, the occurrence and spatial distribution of 58 pharmaceuticals and 19 metabolites and/or TPs (seven of them, with known remaining pharmacological activity) are studied at 24 sampling sites along the Ebro river basin. Relations between the presence of parent pharmaceuticals and their derivates (both metabolites and TPs) have been established.

2. Material and methods

2.1. Study site

Ebro river is located in the northeast of the Iberian Peninsula (see Fig. 2). It drains a total area of 85,550 km², of which 84,415 km² are in Spanish territory. The Ebro river, which is the most important river in Spain, flows 928 km in NW–SE direction into the Mediterranean Sea, where it creates a delta of 320 km², one of the largest wetland areas in the western Mediterranean region. Approximately 2,800,000 people

Fig. 2. Sampling map.

live in the river basin area. The Ebro river basin is largely regulated by dams and channels, which have altered its hydrological and sedimentary regime. Like the rest of Mediterranean rivers, it presents a significant temporal variability, which, together with the increasing industrial and agricultural activity, anthropogenic manipulation of the river hydrology and intensification of climate change, results in serious problems due to an imbalance between the availability of water resources during extended droughts and the increasing anthropogenic water demands. These problems affect not only the water availability, but also the water quality and ecosystem services.

Twenty four sampling locations were selected along the basin to depict a contamination gradient. Fifteen belonged to the tributaries and 9 to the main Ebro river (see Table 1). The sites cover all the strategic points along the basin, including sites near the river sources, like OCA, ESE, CIN1, GAL1 and especially EBR1 and RS; others situated downstream of important urban and industrial areas like Miranda de Ebro (EBR2), Gasteiz (ZAD), Logroño (EBR4), Pamplona (ARG), Tudela (EBR5), Zaragoza (EBR6 and HUE) and Lleida (SEG); NAJ is in an important agricultural wine area and, some other sites were selected in the delta at the mouth of the main Ebro river (EBR8 and EBR9).

2.2. Sample collection

One grab sample of river water was collected at each sampling site in September/October 2010, using amber glass bottles, pre-rinsed with ultra-pure water. After collection and during shipment, samples were kept in the dark, and cooled with ice. Upon reception in the laboratory, samples were vacuum filtered through 1 μm glass fibre filters from Whatman (Fairfield, CT, USA), followed by 0.45 μm nylon

Table 1
Sampling sites.

Sampling site	Locality (province)	River[a]	Average flow (m^3/s)	Sources of contamination/strategic situation
EBR1	Nestares/Reinosa (Cantabria)	Ebro	1.03	Near the source of the river
OCA	Oña (Burgos)	*Oca*	1.30	Near the source of the river
EBR2	Miranda de Ebro (Burgos)	Ebro	18.63	Downstream to industrial/urban area
ZAD	Villodas (Álava)	*Zadorra*	2,05	Downstream to industrial/urban area
EBR3	Haro (La Rioja)	Ebro	–	Intermediate sampling point
NAJ	San Asensio (La Rioja)	*Najerilla*	5.82	Agricultural area
ARG	Echauri (Navarra)	*Arga*	3,18	Downstream to industrial/urban area
EBR4	Mendavia (Navarra)	Ebro	26.13	Downstream to industrial/urban area
EBR5	El bocal–Tudela (Navarra)	Ebro	–	Downstream to industrial/urban area
GAL1	Jabarrella (Huesca)	*Gállego*	10.41	Near the source of the river
GAL2	Villanueva de Gállego (Zaragoza)	*Gállego*	11.25	Before joining the Ebro river
HUE	Fuente de la Junquera (Zaragoza)	*Huerva*	1.81	Downstream to industrial/urban area
EBR6	Presa de Pina (Zaragoza)	Ebro	78.23	Downstream to industrial/urban area
MAR	Alcaine (Teruel)	*Martín*	0.24	Agricultural area
ESE	Graus (Huesca)	*Ésera*	–	Near the source of the river
CIN1	El Grado (Huesca)	*Cinca*	–	Near the source of the river
CIN2	Monzón (Zaragoza)	*Cinca*	–	Intermediate sampling point
RS	Lladurs (Lleida)	*Ribera Salada*	–	Near the source of the river
SEG	Torres de Segre (Lleida)	*Segre*	25.49	Downstream to industrial/urban area
MAT	Nonaspe/Fabara (Zaragoza)	*Matarraña*	0.03	Before joining the Ebro river
ALG	Batea (Tarragona)	*Algars*	–	Before joining the Ebro river
EBR7	Ascó (Tarragona)	Ebro	194.63	Mouth of Ebro river
EBR8	Tortosa (Tarragona)	Ebro	128.64	Mouth of Ebro river
EBR9	Deltebre (Tarragona)	Ebro	–	Mouth of Ebro river

[a] In italics, sites in the tributary rivers.

membrane filters from Teknokroma (Barcelona, Spain), and stored in amber-polyethylene terephtalate (PET) bottles in the dark at −20 °C until analysis.

2.3. Chemicals

All pharmaceuticals and metabolites or TP standards were of high purity grade (>90%) and are listed in the Supplementary data 1.

Both individual stock standard and isotopically labelled internal standard solutions were prepared on a weight basis in methanol (MeOH), except fluoroquinolones which were dissolved in a H_2O/MeOH mixture (1:1) containing 0.2% v/v hydrochloric acid, as they are slightly soluble in pure MeOH. After preparation, standards were stored at −20 °C. Stock solutions were renewed every 3 months. On the other hand, compounds with numbers (see Table 2) 69, 72, 73, and 74 were obtained as solutions in acetonitrile (ACN), 20 and 23 were dissolved in MeOH, at a concentration of 1 mg mL^{-1}, and 22 dissolved in a mixture of MeOH/H_2O (1:1, v/v). A mixture of all pharmaceuticals was prepared by the appropriate dilution of individual stock solutions in MeOH/H_2O (25:75, v/v). Working standard solutions, also prepared in the MeOH/H_2O (25:75, v/v) mixture, were renewed before each analytical run. Working solutions were prepared in amber glass vials while standard mixtures were prepared in volumetric flasks wrapped with aluminium foil to prevent exposure to light. A separate mixture of isotopically labelled internal standards, used for internal standard calibration, was prepared in MeOH and further dilutions were also prepared in the MeOH/H_2O (25:75, v/v) mixture.

HPLC grade MeOH, ACN, acetone and H_2O, hydrochloric acid (thereon HCl) 37%, trifluoro acetic acid (TFA) 99.8% and formic acid (thereon FA) 98% were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dehydrate (Na_2EDTA) was 99%, ammonium acetate (thereon NH_4Ac) was 98% and ammonium hydroxide (thereon NH_4OH) was 28%, all of them from Sigma-Aldrich (Steinham, Germany). 2-Propanol 99.9% was obtained from Carlo Erba Reactifs (Val de Reuil, France).

2.4. Selection of target analytes

The selected 77 target compounds, are listed in Table 2. There are 58 pharmaceuticals and 19 metabolites and/or TP, among them 7 metabolites with remaining pharmacologic activity. They belong to different medicinal classes. Those 58 pharmaceuticals have been selected due to their occurrence and ubiquity in the aquatic environment, according to the information found in the scientific literature, as well as their high human consumption in Spain. Regarding their 19 metabolites and TP, the criteria were, primarily, the remaining pharmacologic activity, or the potential one (glucuronides and acetylated derivates). Some TPs without that feature were still included in the method if their corresponding parent pharmaceutical were usually found at high concentrations in the environment, and/or was known for its high toxicity. However, the final criterion was the commercial availability of analytical standards.

The list of target compounds include eight analgesic and anti-inflammatory pharmaceuticals and 2 of their metabolites. One of the metabolites, salycilic acid, is pharmacologically active. It is the product of the metabolism of both anti-inflammatory agents acetyl salicylic acid and salicin. Thus, after the intake of aspirin, 2–30% is excreted like salicylic acid (Mompelat et al., 2009).

Another important group of target compounds included in the method was formed by 5 lipid regulators, fibrates and statins; and 2 of their metabolites, clofibric acid and 2OH-atorvastatin, both of them pharmacologically active. Clofibric acid is actually the active form of clofibrate, which is used as a prodrug. Regarding the 2OH-atorvastatin, it presents a similar pharmacologic activity as the atorvastatin and at least 2% can be found in the urine.

Five parent psychiatric drugs are monitored. From one of them, the antiepileptic carbamazepine, three metabolites, 2OH carbamazepine, 3OH carbamazepine, 10,11-epoxi-carbamazepine, and two TP formed in wastewater treatment, acridone and acridin, were monitored. Those three metabolites can also be produced by wastewater treatment (Jelic et al., in press; Miao and Metcalfe, 2003). Only the 10,11-epoxi-carbamazepine is still pharmacologically active. After the oral administration of carbamazepine, 72% of the dose is excreted in the urine and 28% is eliminated in the faeces. Only less than 3% of the drug is excreted unchanged in the urine.

Desmethyl diazepam (also known as nordazepam or nordiazepam) is the main metabolite of diazepam and remains pharmacologically active. Diazepam's other active metabolite includes oxazepam which is conjugated with glucuronide. Most of the drug is metabolised; very little diazepam is excreted unchanged, but mainly as oxazepam conjugate (~33%) in the urine. The glucuronization process inactivates the oxazepam, but potential activity remains since the glycosidic bond is very labile and prone to turn back into the active oxazepam during wastewater treatment or in the environment.

Five antihistaminics and one of their active metabolites are included. Urine is the main route of excretion for ranitidine. Approximately 30% of the orally administered dose is excreted as an unchanged drug in 24 h and N-oxide amounts to less than 6% of the dose (Mompelat et al., 2009).

The antibiotics covered by the method include 20 compounds, including 4 metabolites. Anhydroerythromycin arises from the acid-catalysed degradation of erythromycin. It has a negligible antibacterial activity, but inhibits drug oxidation in the liver, and is responsible for unwanted drug-to-drug interactions (Hassanzadeh et al., 2006). The N-acetyl derivates of the sulfamides, i.e., sulfamethoxazole, sulfadiazine and sulfamethazine, are also included. They can easily release the sulfamide drug back, similar to the glucuronides (Göbel et al., 2007). In addition, acetylated forms even though they do not have pharmacological activity, they present ecotoxicity (Eguchi et al., 2004).

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Table 2
Range (minimum and maximum) and mean concentration in ng L^{-1} for target compounds.

Therapeutic group	No	Compound		Pharmacologic activity	CAS number	Concentration (ng L^{-1})						Frequency (%)	
		Pharmaceutical	Metabolite/transformation product			Minimum		Maximum		Mean[a]			
						Ebro	Tributaries	Ebro	Tributaries	Ebro	Tributaries	Ebro	Tributaries
Analgesics/anti-inflammatories and metabolites (10)	1	Naproxen		Yes	22204-53-1	n.d.	n.d.	101	80.5	20	18.8	67	73
	2	Indomethazine		Yes	53-86-1	n.d.	n.d.	bloq.	0.6	n.d.	0.04	11	20
	3	Diclofenac		Yes	15307-86-5	n.d.	n.d.	bloq.	260	n.d.	17.4	22	27
	4		4OH diclofenac	No	64118-84-9	n.d.	n.d.	7.76	48.2	1.13	5.87	44	27
	5	Mefenamic acid		Yes	61-68-7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	6	Acetaminophen		Yes	103-90-2	n.d.	n.d.	712	n.d.	96.5	n.d.	33	0
	7		Salicylic acid	Yes	69-72-7	bloq.	bloq.	73.1	89.1	35.6	42.5	100	100
	8	Propyphenazone		Yes	479-92-5	bloq.	bloq.	4.22	34.7	1.41	3.42	100	100
	9	Phenylbutazone		Yes	50-33-9	n.d.	n.d.	50.9	149	5.65	14.4	11	20
	10	Phenazone		Yes	60-80-0	n.d.	n.d.	bloq.	37.5	n.d.	3.74	22	20
Lipid regulators and metabolites (7)	11		Clofibric acid	Yes	882-09-7	n.d.	n.d.	bloq.	14.4	n.d.	2.25	11	60
	12	Bezafibrate		Yes	41859-67-0	n.d.	bloq.	2.97	51.3	0.45	4.05	56	100
	13	Fenofibrate		Yes	49562-28-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	14	Gemfibrozil		Yes	25812-30-0	n.d.	n.d.	60.4	113	16.2	25.2	33	40
	15	Pravastatin		Yes	81093-37-0	n.d.	n.d.	75.5	7.73	8.4	0.78	22	13
	16	Atorvastatin		Yes	134523-00-5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	17		2OH atorvastatin	Yes	214217-86-4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
Psychiatric drugs, metabolites and transformation products (12)	18	Paroxetine		Yes	61869-08-7	n.d.	n.d.	7.28	7.76	0.81	3.96	11	53
	19	Fluoxetine		Yes	54910-89-3	n.d.	n.d.	14.5	14.5	4.72	3.81	33	27
	20	Diazepam		Yes	439-14-5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	21		Desmethyl diazepam	Yes	1088-11-5	n.d.	n.d.	1.36	3.99	0.15	0.27	11	7
	22		Oxazepam glucuronide	No	6801-81-6	n.d.	n.d.	48.8	70.9	22.7	12.9	78	87
	23	Lorazepam		Yes	846-49-1	n.d.	n.d.	17.4	50.2	3.84	9.19	22	27
	24	Carbamazepine		Yes	298-46-4	2.26	bloq.	11.9	90.4	5.93	13.4	100	100
	25		2OH carbamazepine	No	68011-66-5	n.d.	n.d.	5.38	61.7	1.27	7.46	89	93
	26		3OH carbamazepine	No	68011-67-6	n.d.	n.d.	4.57	70	1.45	9.17	89	73
	27		10,11-epoxi carbamazepine	Yes	36507-30-9	n.d.	n.d.	214	1667	76.5	212	89	80
	28		Acridone	No	578-95-0	n.d.	n.d.	17.5	n.d.	1.94	n.d.	11	0
	29		Acridin	No	260-94-6	n.d.	n.d.	9.64	16.8	2.11	3.29	78	27
Antihistaminics and metabolite (5)	30	Loratadine		Yes	79794-75-5	n.d.	n.d.	3.96	17.1	0.44	1.14	11	13
	31	Famotidine		Yes	76824-35-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	32	Ranitidine		Yes	66357-35-5	n.d.	n.d.	75.2	136	17.6	31.1	89	93
	33		Ranitidine N-oxide	No	73857-20-2	n.d.	n.d.	89.5	196	17.1	32	33	67
	34	Cimetidine		Yes	51481-61-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
Antibiotics and metabolites (20)	35		Anhydroerythromycin	No	23893-13-2	n.d.	n.d.	n.d.	9.5	n.d.	0.7	0	13

	36	Azithromycin		Yes	83905-01-5	n.d.	n.d.	41.1	33.4	4.57	4.4	78	80
	37	Roxithromycin		Yes	80214-83-1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	38	Clarithromycin		Yes	81103-11-9	8.19	6.12	17.2	141	12.1	22	100	100
	39	Josamycin		Yes	16846-24-5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	40	Sulfamethoxazole		Yes	723-46-6	n.d.	n.d.	3.03	17.2	0.51	1.39	33	20
	41		N-acetyl sulfamethoxazole	No	21312-10-7	n.d.	n.d.	n.d.	73.8	n.d.	5.21	0	13
	42	Sulfadiazine		Yes	68-35-9	bloq.	39.7	51	136	39.7	50.9	100	100
	43		N-acetyl sulfadiazine	No	127-74-2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	44	Sulfamethazine		Yes	57-68-1	n.d.	n.d.	55.5	641	7.96	53.1	56	60
	45		N-acetyl sulfamethazine	No	100-90-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	46	Ofloxacin		Yes	82419-36-1	n.d.	n.d.	78.6	79.9	8.74	10.2	22	40
	47	Ciprofloxacin		Yes	85721-33-1	n.d.	n.d.	bloq.	n.d.	n.d.	n.d.	11	7
	48	Norfloxacin		Yes	70458-96-7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	49	Danofloxacin		Yes	112398-08-0	n.d.	n.d.	bloq.	bloq.	n.d.	n.d.	11	33
	50	Enoxacin		Yes	74011-58-8	n.d.	n.d.	n.d.	bloq.	n.d.	n.d.	0	13
	51	Flumequine		Yes	42835-25-6	n.d.	n.d.	bloq.	bloq.	n.d.	n.d.	22	7
	52	Trimethoprim		Yes	738-70-5	bloq.	n.d.	12	59.9	3.96	9.46	100	80
	53	Chloramphenicol		Yes	56-75-7	n.d.	n.d.	bloq.	bloq.	n.d.	n.d.	11	7
	54	Metronidazole		Yes	443-48-1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
Cardiovascular drugs and metabolites (15)	55	Atenolol		Yes	29122-68-7	n.d.	n.d.	11.2	475	2.16	39.3	33	33
	56	Sotalol		Yes	3930-20-9	n.d.	n.d.	bloq.	bloq.	bloq.	bloq.	56	53
	57	Metoprolol		Yes	37350-58-6	n.d.	n.d.	6.52	14.8	5.52	6.33	89	93
	58	Propranolol		Yes	525-66-6	bloq.	bloq.	9.89	18.82	6.18	8.96	100	100
	59		Propranolol-β-D-glucuronide	No	NA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	60	Timolol		Yes	26839-75-8	n.d.	n.d.	3.45	3.63	1.8	2	67	80
	61	Betaxolol		Yes	63659-18-7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	62	Carazolol		Yes	57775-29-8	n.d.	bloq.	9.23	8.39	5.11	6.97	78	100
	63	Pindolol		Yes	13523-86-9	n.d.	n.d.	10.9	81	4.71	10	78	87
	64	Nadolol		Yes	42200-33-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
	65	Enalapril		Yes	75847-73-3	n.d.	n.d.	2.91	2.3	0.67	1.05	33	60
	66		Enalaprilat	Yes	84680-54-6	bloq.	n.d.	11.5	12.9	7.32	6.23	100	87
	67	Hydrochlorothiazide		Yes	58-93-5	n.d.	10.9	190.1	494	79.1	144	89	100
	68	Lisinopril		Yes	83915-83-7	n.d.	n.d.	24.2	116	16	19	78	60
	69	Furosemide		Yes	54-31-9	n.d.	n.d.	bloq.	108	n.d.	7.23	11	20
β-agonists (2)	70	Albuterol		Yes	18559-94-9	n.d.	n.d.	n.d.	1.43	n.d.	0.1	0	7
	71	Clenbuterol		Yes	37148-27-9	n.d.	n.d.	bloq.	bloq.	n.d.	n.d.	11	33
Barbiturates (3)	72	Butalbital		Yes	77-26-9	n.d.	n.d.	n.d.	bloq.	n.d.	n.d.	0	7
	73	Pentobarbital		Yes	76-74-4	n.d.	n.d.	bloq.	n.d.	n.d.	n.d.	22	0
	74	Phenobarbital		Yes	50-06-6	n.d.	n.d.	31.9	99.5	3.55	19.3	33	67
Antidiabetic and metabolite (2)	75	Glyburide		Yes	10238-21-8	n.d.	n.d.	96.74	n.d.	10.75	n.d.	11	0
	76		Trans 4OH glyburide	Yes	23155-00-2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0	0
Antineoplastic (1)	77	Tamoxifen		Yes	10540-29-1	12.4	22.4	20.1	26.8	18.9	22.7	100	100

[a] Values below the limit of detection and below the limit of quantification were considered 0 to calculate the mean.

under ESI pos, or 10 mM NH_4Ac for the aliquot analysed by ESI neg) and 2% MeOH (to minimise the loss of the analytes through adsorption onto the surface of the glassware), and the fortification with a surrogate mixture at 100 ng L^{-1} to correct for potential losses during the analytical procedure, as well as, for matrix effects.

The MS/MS detection was performed by a Thermo Scientific TSQ Vantage™ triple quadrupole mass spectrometer (MS) with an ESI source (Waltham, MA, USA). Data acquisition was performed in the selected reaction monitoring (SRM) mode recording the transitions between the precursor ion and the two most abundant product ions for each target analyte. SRMs are shown, along with the optimised compound-dependent S-lens RF amplitude and collision energy (CE), in Supplementary data 2. Recoveries were higher than 80% for majority of the studied compounds and method limits of detection were under 10 ng L^{-1} for 75% of analytes in surface water. Fourty-six isotopically labelled compounds were used as surrogates, and the assignation can also be seen in Suppementary data 2. Other experimental conditions and optimization parameters are described in details in López-Serna et al. (in press).

3. Results and discussion

The quantified levels (concentration range and mean values) and the frequency of detection of the investigated pharmaceuticals and transformation-products in the water samples analysed, are summarised in Table 2.

Several previous studies (Gros et al., 2007, 2010; López-Serna et al., 2011) determined the levels of pharmaceuticals in the Ebro river basin, but none of them included metabolites or TPs. In fact, very few works worldwide have been published reporting concentrations of pharmaceutical TP in surface waters (González Alonso et al., 2010; Huerta-Fontela et al., 2011; Langford and Thomas, 2011; Letzel et al., 2010; Martínez Bueno et al., 2007; Yu et al., 2011). This shows that the available data about the presence of pharmaceutical metabolites and TP in the aquatic environment is very scarce. In addition, the number of metabolites and TP included in previously published studies was generally low, limited to only several compounds (Langford and Thomas, 2011). In most of the cases, metabolites and/or TP, belonging to the psychiatric therapeutic class, especially, metabolites of carbamazepine and diazepam were included in the methods.

3.1. Levels of pharmaceuticals, their metabolites and TPs

Out of the 77 target compounds monitored, 7 (the analgesic propyphenazone, the psychiatric drug carbamazepine, the antibiotics clarithromycin and sulfadiazine, the cardiovascular drug propranolol, the antineoplastic tamoxifen and 1 pharmacologically active metabolite salicylic acid) were found to be ubiquitous (detected in all samples). The concentration of tamoxifen was lower than that found by Roberts and Thomas (2006), but higher than that reported for influent sewage water samples by Liu et al. (2010) and Tauxe-Wuersch et al. (2006). Another 53 compounds, including 14 metabolites and TPs were detected in at least in one sample (see Table 2). In general, concentrations found were lower than 100 ng L^{-1}, with several compounds showing higher concentrations in some samples. Highest individual and also highest mean concentration was detected for a metabolite (and TP) of carbamazepine 10,11 epoxi carbamazepine that was detected in more than 80% of the samples with a mean concentration of 76.5 ng L^{-1} in the Ebro river and 211.6 ng L^{-1} in smaller tributary rivers. The highest concentration of 1667 ng L^{-1} was detected in a small tributary, Zadorra river (sampling site ZAD, flow 1.4 m^3/s) that is impacted by discharges from the city of Vitoria (Gasteiz). These levels are much higher than previously reported in the Llobregat river (NE Spain) where an average concentration of 54 ng L^{-1} was found by Huerta-Fontela et al. (2011) or 35.6 ng L^{-1} in the surface water of Oslofjord (Norway) as reported by Langford and Thomas (2011). Other compounds, detected at levels higher than 200 ng L^{-1}, were sulfamethazine, atenolol, hydrochlorothiazide and diclofenac. Besides the above mentioned 10,11 epoxi carbamazepine, other metabolites and TP detected were ranitidine N-oxide (maximum concentration 196 ng L^{-1}), oxazepam glucoronide (maximum concentration 70.9 ng L^{-1}), 4OH-diclofenac (maximum concentration 48.2 ng L^{-1}), acridin (maximum concentration 16.8 ng L^{-1}) and N-acetyl sulfamethoxazole (maximum concentration 73.8 ng L^{-1}). The mean concentration of desmethyl diazepam was 0.22 ng L^{-1} which was significantly lower than the values reported for surface waters in the metropolitan area of Madrid, Spain (González Alonso et al., 2010), with a mean concentration of 33 ng L^{-1}.

Seventeen target compounds were not detected in any of the sampling locations. Twelve of them were parent compounds, i.e., mefenamic acid, fenofibrate, atorvastatin, diazepam, famotidine, cimetidine, roxithromycin, josamycin, norfloxacin, metronidazole, betaxolol and nadolol and 5 metabolites, i.e., N-acetyl sulfadiazine, N-sulfamethazine, propranolol-β-D-glucuronide, trans 4OH glyburide and 2OH atorvastatin.

3.2. Contribution of metabolites/TPs to the overall load of pharmaceuticals

Concentrations of metabolites and TPs detected in this study were compared to the concentrations of their corresponding parent compound in order to estimate and evaluate the contribution of metabolites and TPs to the overall burden of pharmaceuticals in the aquatic environment. The total concentration of all detected parent compounds (58 compounds) and the total concentration of all detected metabolites/TPs (19 compounds) are shown in Fig. 3A and B. Although the number of monitored metabolites/TPs was significantly lower than the number of parent compounds and not all possible metabolites/TPs are included into the method the overall contribution of metabolites/TPs is found to be significant. In all sampling points metabolites/TPs are found to be present at the same concentration level as the parent compounds, representing as an average 30–50% of the total pharmaceutical load (parent compounds + metabolites/TPs).

For individual compounds, mean concentrations for most of the metabolites and TPs were found to be at a similar concentration level as their corresponding parent compound. Fig. 4A shows the levels found for ranitidine and its metabolite ranitidine N-oxide that were detected in 19 out of 24 samples at average concentrations of 24 ng L^{-1} for ranitidine and 24.5 ng L^{-1} for ranitidine N-oxide. However, the most interesting case is carbamazepine and its metabolites (and/or TPs). As shown in Fig. 4B. the concentration of metabolites/TPs significantly surpassed the concentration of the parent compound in all samples, being that the individual concentration of 10,11-epoxi carbamazepine was almost one order of magnitude higher that the concentration of carbamazepine. Similar results were reported by Langford and Thomas (2011) and Huerta-Fontela et al. (2011), although the ratio metabolite/carbamazepine was 5 and 4, respectively, compared to this study where the ratio was between 10 and 15. 10,11-Epoxi carbamazepine has been found at higher concentrations than its parent pharmaceutical in all the published works so far. But the ratio metabolite vs. pharmaceutical was around 5, while in our study the concentration of that active metabolite was 15 times higher than that of its parent compound carbamazepine. Desmethyl diazepam was also found at higher concentrations than its parent diazepam in González Alonso et al. (2010).

All this shows the importance of monitoring transformation-products of pharmaceuticals, especially those ones which remain pharmacologically active. The presence of metabolites in the environment is not surprising since a high percentage of the dose is excreted as metabolites for most of the pharmaceuticals. In fact diazepam is excreted in the urine as oxazepam glucuronide (33%) and just traces of desmethyl diazepam and unchanged pharmaceutical. A similar

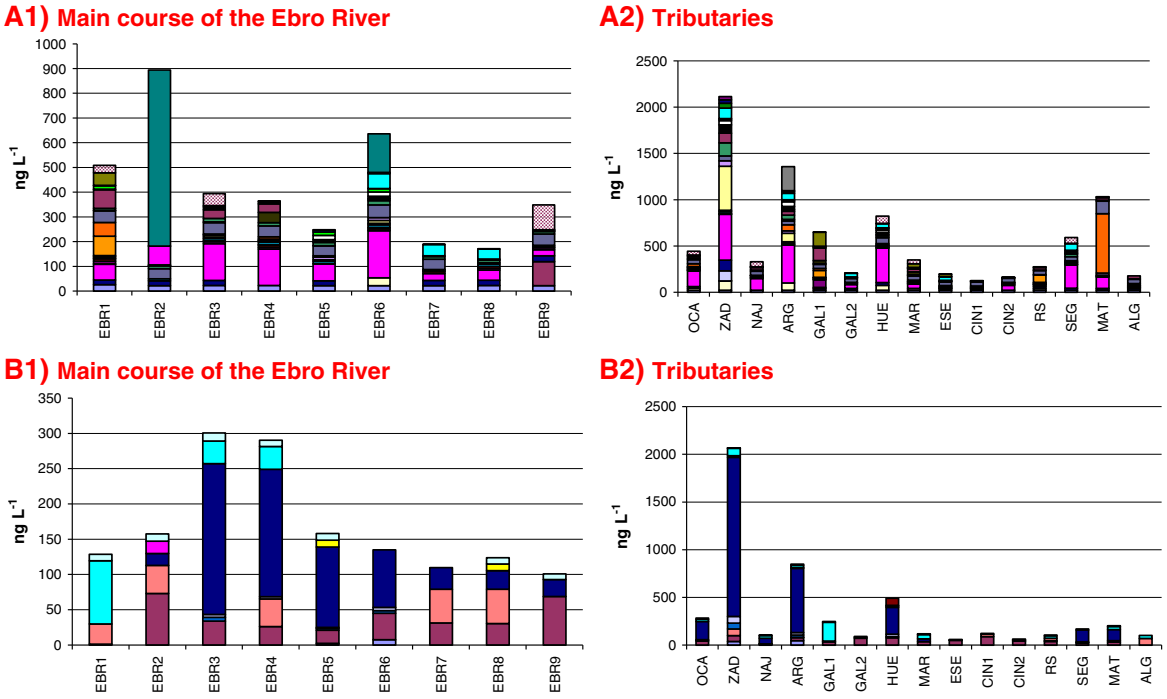

Fig. 3. Sum concentration (ng L^{-1}) of A) 58 parent pharmaceuticals and B) 19 metabolites and/or transformation products detected.

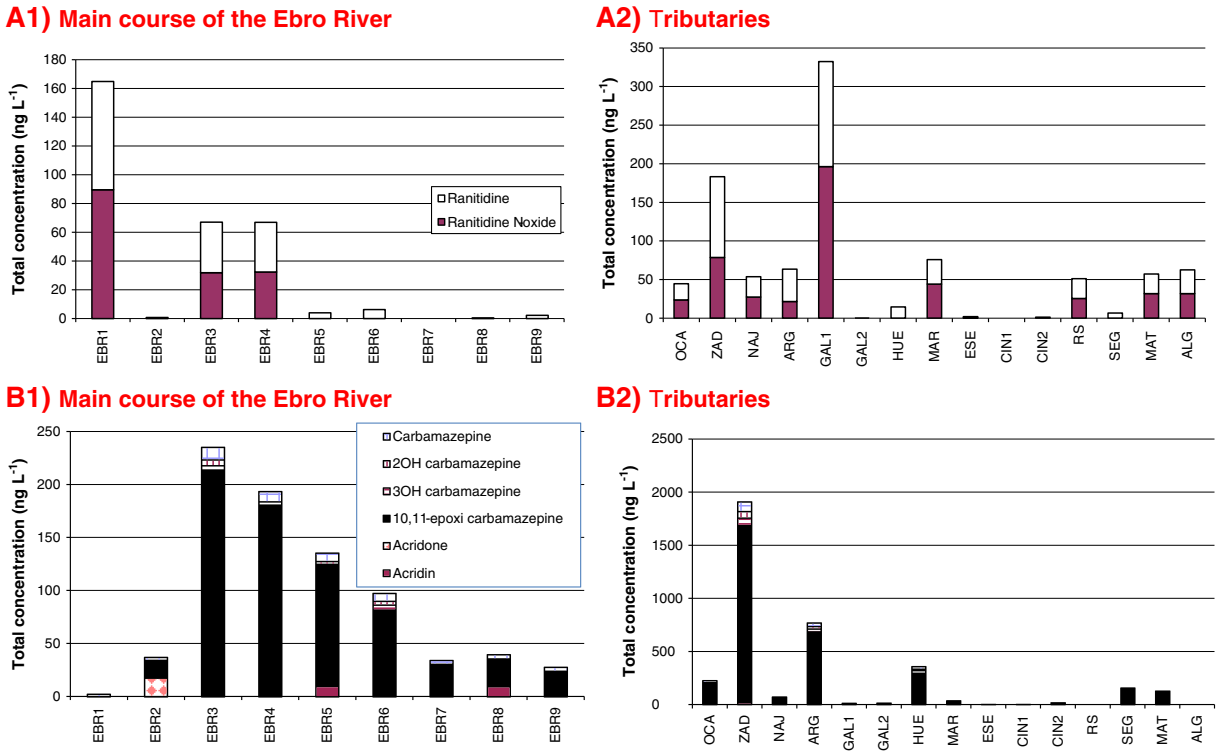

Fig. 4. Sum concentrations (ng L^{-1}) of A) ranitidine and its transformation product B) carbamazepine and its transformation products detected.

ratio was found to be present in environmental waters in the present study. However, for some other compounds like ranitidine and especially for carbamazepine, lower concentrations of their corresponding metabolites, ranitidine N-oxide and 10,11-epoxi carbamazepine, would be expected taking into account the data on excretion (4% and 2%, respectively, versus 30% and 3% of unchanged pharmaceutical, respectively) (Levy et al., 2002). The change in ratios may be attributed to several reasons. First of all, the theoretical percentages of excretions can vary for elderly persons or patients with hepatic dysfunction or any other illness like diabetes, high blood pressure, renal failure, etc. since the metabolism may work differently in them (Simon et al., 1988; Tocco et al., 1982). In addition, pharmaceutical industries or the disposal of expired or no longer needed medicine from the users contribute to the increased finding of unchanged pharmaceuticals in the environment. However, the main factors are the transformations in the WWTPs and/or in the environment that do not affect all compounds to the same extent.

3.3. Spatial distribution

The highest concentration of both parent compounds and metabolites and TPs are found at sites downstream of the big urban centres of Pamplona (ARG), Zaragoza (HUE) and especially Gasteiz (ZAD). In general, higher concentrations of the target compounds were found in small tributary rivers than in the main Ebro river (see Table 2 and Fig. 3). This is attributed to the lower dilution in small tributaries in comparison to the main stream Ebro river (See flows in Table 1). On the contrary, sites GAL2, ESE, CIN1, CIN2, ALG, EBR7 and EBR8 were shown as the less contaminated with a total concentration of all target compounds, below 200 ng L^{-1}. This is not surprising for ESE and CIN1 which correspond to locations near the source of the river. However, EBR7 and EBR8 were situated near the mouth of the main Ebro River, and the low concentrations detected there reflect high dilution factors due to high river flow in the lower part of the river. In fact, the concentration was kept quite similar along the main Ebro River. This means that despite the input of effluents from the WWTPs situated along the river (i.e. cities of Logroño, Tudela, Zaragoza), the dilution phenomenon keeps the concentration stable. In fact, Table 1 shows how Ebro river flow was increasing gradually from near its source (1.03 m^3/s in EBR1) until near its mouth (128.6 m^3/s in EBR8) with a total increase of more than 100 times. Natural attenuation can be also taking place. This was observed in López-Serna et al. (2011) as well.

4. Conclusions

The present work reports the environmental occurrence of 58 pharmaceuticals and 19 metabolites and TPs in the Ebro river basin. In all sampling points metabolites/TPs are found to be present at the same concentration level as the parent compounds, representing as an average 30–50% of the total pharmaceutical load (parent compounds + metabolites/TPs). The highest concentrations are found for the active metabolite of the antiepileptic carbamazepine, 10,11-epoxi carbamazepine that is determined at concentrations more than one order of magnitude higher than that of parent carbamazipine. Concentrations were in general below 100 ng $L^{-}1$, with a few exceptions. 10,11-Epoxi carbamazepine is found at a maximum concentration of 1600 ng L^{-1}. In general, the highest concentrations are found in small tributary rivers receiving effluents from WWTP. In the main Ebro river, the levels are similar from its source to its mouth showing that the dilution effect and the natural depletion counteract the continuous introduction of pharmaceuticals via WWTP effluents and other sources along the river. With all the aforementioned, more studies on the occurrence of metabolites/TPs in environmental waters would be necessary, especially for the ones with remaining pharmacologic activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2012.06.027.

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Publicación científica #7

"Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain"

por:

Rebeca López-Serna, Anna Jurado, Enric Vázquez-Suñé, Jesus Carrera, Mira Petrović, Damià Barceló

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Occurrence of 95 pharmaceuticals and transformation products in urban groundwaters underlying the metropolis of Barcelona, Spain

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ABSTRACT

The present paper presents the occurrence of 72 pharmaceuticals and 23 transformation products (TPs) in groundwaters (GWs) underlying the city of Barcelona, Spain. Thirty-one samples were collected under different districts, and at different depths. Aquifers with different geologic features and source of recharge were included, i.e., natural bank filtration, infiltration from wastewater and water supply pipes, rainfall recharge, etc. Antibiotics were the most frequently found compounds detected at levels reaching 1000 ng L^{-1}. Natural bank filtration from the river that receives large amounts of effluents from waste water treatment plants (WWTPs), turned out being the most influencing source of contamination, thus GW showed high range of compounds and concentrations as high as or even higher than in the river itself. In general, TPs were found at lower concentrations than the corresponding parent compounds, with some exceptions, such as 4OH propranolol and enalaprilat.

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1. Introduction

Groundwater (GW) is an important resource of water supply in the world. In Catalonia, GW represents 35% of the water resources, of which 30–35% is used for drinking water, while the rest is used for industrial activities and agriculture supply (García-Galán et al., 2010). In Barcelona metropolitan area GW represents 19% of all the water resources and currently it is extracted for purposes where quality requirements are less stringent than for human consumption, such as municipal services (watering parks, street cleaning, ornamental fountains, etc.), other uses, including industrial (building heating and cooling and car washing, industrial cleaning, etc.), environmental (regeneration of the Vallvidrera reservoir and the Besòs riverbed) and agricultural.

Urban aquifers are especially vulnerable to contamination by a variety of contaminants due to urban activities and industry. Although soil provides a big inertia to quality changes and slows propagation of the contamination, for that same reason, once contaminated, the effects can hardly ever be reverted (García-Galán et al., 2010, 2011). Previous studies showed that aquifers underlying the Barcelona metropolitan area suffer pollution from different sources, including losses from water supply networks, leakage from sewers, seepage from rivers or other surface water (SW) bodies, and seawater intrusion. Emerging contaminants, such as surfactants and their degradation products (i.e. nonylphenol ethoxylates, nonylphenol carboxylates, nonylphenol and linear alkyl benzene sulfonates) were found at high concentrations (μg/L level) in aquifers recharged by the river Besòs showing clear dependence on the sources of recharge or/and pollution containing these substances and groundwater redox conditions (Tubau et al., 2010). Low, but measurable concentrations of drugs of abuse, are also found in zones recharged by a river that receives large amounts of effluents from WWTPs (Jurado et al., 2012).

Pharmaceuticals are another important class of emerging contaminants that can potentially reach GW. The use of pharmaceuticals by the modern society is huge and steadily increasing. They are developed with the intention of performing a biological effect and they may exert their activity at the low ng L^{-1} range (Vulliet and Cren-Olivé, 2011), therefore harmful effects to the ecosystem and humans should be evaluated (Eggen et al., 2010).

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Furthermore, the metabolites and biotic and abiotic transformation products (TPs) of parent pharmaceuticals can remain pharmacologically active.

Only a few studies of presence of pharmaceuticals in urban GWs (e.g., Grujić et al., 2009; Osenbrück et al., 2007; Reinstorf et al., 2008; Schirmer et al., 2011; Strauch et al., 2008; Wolf et al., 2006) can be found in the literature, and in all cases, very few pharmaceuticals were monitored and none of the studies included TPs. Analgesics and antibiotics were the classes more studied, and the levels found ranged from low ng L^{-1} (Standley et al., 2008; Vulliet and Cren-Olivé, 2011), to hundreds of ng L^{-1} (Grujić et al., 2009; Teijon et al., 2010).

In general, TPs are not frequently monitored, and only a few studies reported levels of in GW, i.e., 5 acetylated TPs of sulphonamides surveyed in Catalonia (region Barcelona belong to) (García-Galán et al., 2011), and TPs of phenazone-type compounds in GW in Germany (Massmann et al., 2008a, 2007, 2008b).

The present work studied the presence of 72 pharmaceuticals and 23 of their TPs in GW underlying the city of Barcelona (Spain). Eight of those 23 TPs are pharmacologically active, and another 8 are potentially active as glucuronides or acetylated derivates of active substances. To the knowledge of the authors, this is the first time such a big number of pharmaceuticals, especially TPs, have been studied in GW so far.

2. Material and methods

2.1. Study site

Fig. 1 shows the study area, situated in the North-East of Spain. The analyzed GWs are situated under 136 km^2 of urban realm, which includes Barcelona metropolitan area, where 2.2 million people live. The area is bounded by the Mediterranean Sea and the Collserola Range. Both, Llobregat and Besòs Rivers, close the rectangular zone. The climate is typically Mediterranean with an average rainfall of 600 mm $year^{-1}$.

Samples belong to different aquifers with different lithologies and ages, and can be divided into three groups. First group, located under the district called Poble Sec (PS), belongs to either Barcelona plain (samples labeled as upper depth (u)), or to the underlying confined aquifer (samples labeled as medium depth (m)). Barcelona plain is cropping out and consists of carbonated clays from Pleistocene, Quaternary. The underlying aquifer consists of sandstones, marls and sands from Miocene, Tertiary. Samples featured as (a) contain water from both aquifers, since the screened depth covers both units. See Table 1. The second group of samples is under Mallorca Street (MS), situated midway between Collserola range and the sea. They belong to either the Barcelona plain (samples labeled as upper depth (u)), or to the underlying confined aquifer (samples labeled as medium (m) and low depth (l)). Again, samples typified as (a) contain water from both aquifers since the depth screened covered both groundwater units. The third group of samples was located under Besòs River Delta (BRD). All of them belong to the most superficial unconfined aquifer, composed of gravels, sands, silts and clays from Holocene, Quaternary. Underlying that, there are another two aquifers with the same composition, confined this time, and separated by lutidic units.

Generally, in all aquifers, GW flows seawards, from the mountain range. Several recharge sources have been identified (Vázquez-Suñé et al., 2010). These include: (1) rainfall infiltration in the non-urbanized areas at the highest parts of the city (Collserola range hillside), which is consider as "clean" natural recharge water; (2) seawater intrusion; (3) Besòs River infiltration, which contains a large proportion of effluents from WWTPs, especially during the summer, when precipitations are scarce; (4) losses from the water supply network; (5) losses from the sewage system; and (6) runoff water from the paved areas, which washes away the urban surface and recharges the aquifers through direct infiltration or sewer seepage. A previous study by Jurado et al. (2012), calculates the proportion in which those sources, contribute to the recharge of every area, when the sampling campaign took place. According to that, in MS area, the main contributor to the total recharge is rainfall infiltration (60%), especially in the deepest aquifer (where GRA-2 was sampled), followed by the losses from sewage system (31%) and the losses from the water supply network (9%). In PS area, the main contributors are the losses from sewage system and losses from the water supply network, accounting for 96% (50% and 46%, respectively). The remaining 4% corresponds to rainfall infiltration. Regarding to BRD area, infiltration of water from the Besòs River is the largest contributor to the total recharge, representing 91%, but other contributors are losses from sewage system and water supply network. When considering all the 3 zones as a whole, average proportions are as follows: 21% corresponds to losses from the water supply network, 28% to losses from the sewage system, 18% to rain infiltration, and 33% to recharge from the Besòs River.

The presence of oxidizing species like nitrate, and the absence of ammonium, indicates oxidizing conditions in MS, and especially in PS. In the latter, nitrate was found at concentrations as high as 150 mg/L, along with high levels of dissolved oxygen. In contrast, BRD showed reducing conditions.

2.2. Sample collection

Thirty-one GW samples and one sample from Besòs River were collected during three field campaigns in May 2010 (27 GW and 1 Besòs River), December 2010 (1 GW) and May 2011 (three GW). See Supplementary data 1. Out of the 30 samples,

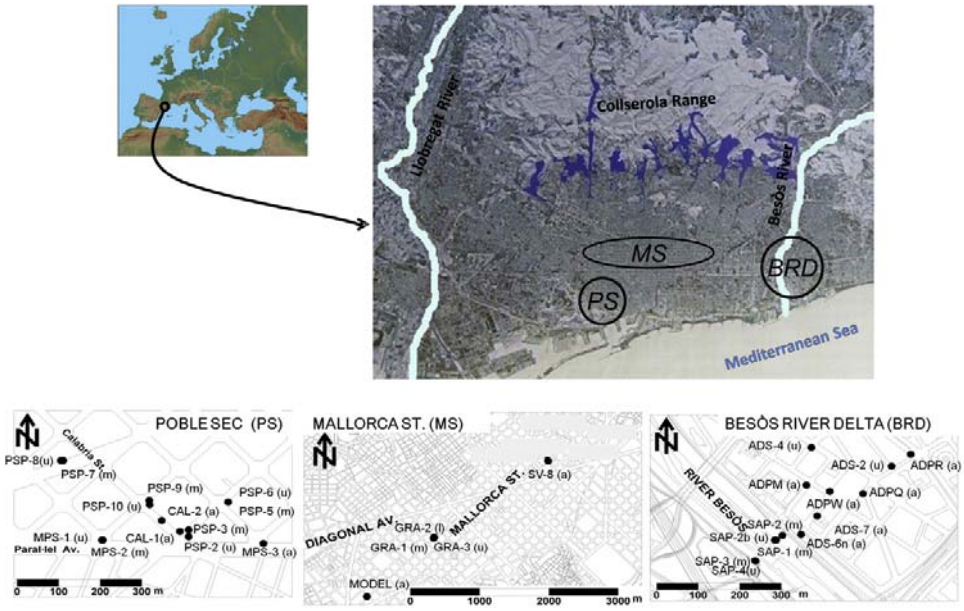

Fig. 1. Sampling map.

Table 1
Sampling sites.

Zone	Sample	Approx. ground height above sea level (m)	Screen depth (m)		Screen height above sea level (m)[a]		Label[b]	Lithology	Material
			Upper screen depth	Low screen depth	Upper screen height	Low screen height			
PS	**PSP-2**	12.8	13	19	−0.2	−6.2	**u**	Clay	Fine
	PSP-3	13.1	23	27	−9.9	−13.9	**m**	Sand	Thick
	PSP-5	12	21	25	−9	−13	**m**	Sand	Thick
	PSP-6	12	16	20	−4	−8	**u**	Clay	Fine
	PSP-7	14	23	27	−9	−13	**m**	Sand	Thick
	PSP-8	14	13	19	1	−5	**u**	Clay	Fine
	PSP-9	12.2	24	26	−11.8	−13.8	**m**	Sand	Thick
	PSP-10	12.5	19	23	−6.5	−10.5	**u**	Clay	Fine
	MPS-1	14.8	11.5	16	3.3	−1.2	**u**	Clay	Fine
	MPS-2	14.7	25	32	−10.3	−17.3	**m**	Sand	Thick
	MPS-3	11.8	6	27	5.8	−15.2	**a**	–	–
	CAL-1	13.1	–	–	–	–	**a**	–	–
	CAL-2	13	–	–	–	–	**a**	–	–
MS	**SV-8**	26	11.5	38	14.5	−12	**a**	–	–
	GRA-1	35	28.5	30	6.5	5	**m**	Sand in clay matrix	Thick
	GRA-2	35	38	41	−3	−6	**l**	Sand	Thick
	GRA-3	35	24	28	11	7	**u**	Sand and gravel	Thick
	MODEL	32	13	37	19	−5	**a**	–	–
BRD	**ADS-2**	7.97	–	–	–	–	**u**	Sand and gravel	Thick
	ADS-4	8.9	–	–	–	–	**u**	Sand and gravel	Thick
	ADS-6N	8.4	3	15	5.4	−6.6	**a**	Sand and gravel	Thick
	ADS-7	8.56	3	15	5.56	−6.44	**a**	Sand and gravel	Thick
	ADPM	8.75	10.5	17.5	−1.75	−8.75	**a**	Sand and gravel	Thick
	ADPR	8.2	9.5	16.5	−1.3	−8.3	**a**	Sand and gravel	Thick
	ADPQ	8.05	10.5	17.4	−2.45	−9.35	**a**	Sand and gravel	Thick
	ADPW	7.1	11	15	−3.9	−7.9	**a**	Sand and gravel	Thick
	SAP-1	5.25	9.5	11.5	−4.25	−6.25	**m**	Sand and gravel	Thick
	SAP-2	5.5	9.5	11.5	−4	−6	**m**	Sand and gravel	Thick
	SAP-2b	5.25	4.5	6.5	0.75	−1.25	**u**	Sand and gravel	Thick
	SAP-3	5	10.5	12.5	−5.5	−7.5	**m**	Sand and gravel	Thick
	SAP-4	5	5.5	7.5	−0.5	−2.5	**u**	Sand and gravel	Thick
	BESÒS	4	–	–	–	–	**–**		

[a]Relative situation for the screen, respecting the sea level.
[b]u: upper screen depth; m: middle screen depth; l: lower screen depth; a: totally screened.

9 belong to pumping wells (MPS-1, MPS-2, MPS-3, CAL-1 and CAL-2 in PS, and ADPM, ADPR, ADPQ and ADPW in BRD) and 21 to observation piezometers. The location of all them and their screen depths and heights above sea level are displayed in Table 1, respectively. Besòs River water, which is the main source of recharge in BRD aquifers, was collected in one sampling site within the BRD area. The purpose of that sample was to show a rough estimation which could be used as a comparative element in that area. Thus, one grab sample was considered sufficient. GW samples were obtained after pumping a volume of at least three times that of the piezometer. Some field parameters were measured in situ and in continuous by using a flow cell to avoid contact to air. They included electrical conductivity, pH, temperature, Eh and dissolved oxygen. The instruments used for those measures were calibrated daily. All samples were collected in amber-polyethylene terephtalate (PET) bottles after stabilization of the field parameters. Subsequently, they were kept in the dark, cooled inside chambers with ice until transported to the laboratory. Once there, samples were filtered and stored in the dark at −20 °C until analysis.

2.3. Chemicals

All pharmaceutical and TP standards were of high purity grade (>90%) and are listed in the Supplementary data 2.

Both individual stock standard and isotopically labeled internal standard solutions were prepared on a weight basis in methanol (MeOH), except fluoroquinolones which were dissolved in a H_2O/MeOH mixture (1:1) containing 0.2% v/v hydrochloric acid, as they are slightly soluble in pure MeOH. After preparation, standards were stored at −20 °C. Special precautions have to be taken into account for tetracycline antibiotics, which have to be stored in the dark in order to avoid their exposure to the light, since it has been demonstrated that they are liable to photodegradation (Eichhorn and Aga, 2004). Fresh stock solutions of antibiotics were prepared monthly due to their limited stability while stock solutions for the rest of substances were renewed every 3 months. Working standard solutions, prepared in MeOH/H_2O (25:75, v/v) mixture, were renewed before each analytical run. A separate mixture of 53 isotopically labeled internal standards, used for internal standard calibration, was prepared in MeOH and further diluted in MeOH/H_2O (25:75, v/v).

HPLC grade MeOH, ACN and H_2O, hydrochloric acid (HCl) 37%, and formic acid (FA) 98% were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA) was 99% from Sigma–Aldrich (Steinham, Germany).

2.4. Selection of target analytes

The selected 95 target compounds, belonging to different medicinal classes, i.e., 14 analgesics, 8 lipid regulators, 12 psychiatric drugs, 5 antihistaminics, 31 antibiotics, 16 cardiovascular drugs, 2 β-agonists, 3 barbiturates, 3 antidiabetics and 1 antineoplastic, are listed in Table 2. There are 72 pharmaceuticals and 23 TPs, among them 8 with remaining pharmacologic activity (salicylic acid, clofibric acid, desmethyldiazepam, 10,11-epoxi carbamazepine, 4OH propranolol, enalaprilat, cis 3OH glyburide, trans 4OH glyburide) and another 8 are potentially active as glucuronides or acetylated derivates of active pharmaceuticals or TPs (Diclofenac Acyl-β-D-glucuronide, 2OH atorvastatin Acyl-β-D-glucuronide, Oxazepam glucuronide, N-acetyl sulfamethoxazole, Sulfamethoxazole-β-D-glucuronide, N-acetyl sulfadiazine, N-acetyl sulfamethazine, Propranolol-β-D-glucuronide). Glucuronization is a metabolic process used by the body to make a large variety of substances more water-soluble, and, in this way, allow for their subsequent elimination from the body through urine or faeces. That process links the target substance to a molecule of glucuronic acid by a glycosidic bond. Normally, pharmaceuticals and active TPs inactivate after that reaction. But, resulting glucuronides might remain potential activity because the glycosidic bond is very labile, i.e., it needs very low energy to get broken, and therefore, it can turn into the active substance in the treatment plants or in the environment. Similarly, N-acetyl derivates can also easily deconjugate (Göbel et al., 2007).

2.5. Analytical method

The analyses of the target compounds in the collected samples, were performed following a previously published, fully automated method based on on-line Solid Phase Extraction–Liquid Chromatography–Electrospray–Tandem Mass Spectrometry (SPE–LC–ESI–MS/MS) (López-Serna et al., 2010), readily adapted for the analysis of the TPs. Method recovery data for HPLC grade water, GW and SW can be found in Table 3. In brief, for the preconcentration and cleanup, samples underwent a fully automated SPE with the aid of a Symbiosis Pico system (Spark Holland, Emmen, The Netherlands) which was coupled on-line to the LC–MS/MS system.

The analytical procedure consisted of two sample injections, both of 2.5 mL in the positive and negative electrospray mode (ESI pos and ESI neg, respectively). In this fully automated methodology, the sample handling was limited to the filtration of the raw water sample through 0.45 μm, the addition of Na_2EDTA at 0.1% (m/v) and the fortification with a mixture of 53 isotopically labeled pharmaceuticals at 100 ng L^{-1}. In every injection, sample is preconcentrated onto previously conditioned polymeric HySphere Resin GP cartridges from Spark Holland (Emmen, The Netherlands). Then, after washing the cartridges with HPLC water, the retained compounds are eluted to the LC–MS/MS system with the chromatographic mobile phase, which for ESI pos, consists of a gradient ACN/0.1% (v/v) formic acid and, for ESI neg, a ACN:MeOH (1:1, v/v)/H_2O gradient. Chromatographic separation is carried out in a Purospher Star RP-18 (125 mm × 2.0 mm, particle size 5 μm), an end-caped analytical column from Merck (Darmstadt, Germany). Detection is performed recording two selected reaction monitoring (SRM) transitions per analyte and one SRM per surrogate; by a 4000 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer from Applied Biosystems-Sciex (Foster City, California, USA). Full list of SRMs and instrumental conditions are given in Supplementary data 3. Quantitation, based on peak areas, is carried out by internal standard approach. See the assignment of surrogates in Supplementary data 3.

3. Results and discussion

3.1. Occurrence of pharmaceuticals and TPs

The quantified levels (average and concentration range) and the frequency of detection of the investigated pharmaceuticals and TPs in the GW samples analyzed, distinguishing every area (PS, MS and BRD), are listed in Table 2.

Out of the 95 target compounds monitored, 11 were found ubiquitous in the GW samples. Among them, 9 pharmaceuticals, the analgesic mefenamic acid, the lipid regulator fenofibrate, the antibiotics clarithromycin, ofloxacin, enoxacin and enrofloxacin, the cardiovascular drugs metoprolol and hydrochlorothiazide, and the antineoplastic tamoxifen; and 2 TP, the pharmacologically active ones, salicylic acid and 4OH propranolol. Another 73 compounds, including 14 TPs were detected at least in one sample (see Table 2).

Average concentrations were generally far below 100 ng L^{-1} in the majority of samples, but several individual concentrations surpassed that value, especially of analgesics, antibiotics and diuretics. The highest concentrations, surpassing 1 μg L^{-1} in some case, were found for the macrolide antibiotics azithromycin and spiramycin, while anti-inflammatory drugs, such as ibuprofen and diclofenac were found at maximum concentrations of 988 and 380 ng L^{-1} respectively. Diuretic hydrochlorothiazide is found at maximum concentration of 665 ng L^{-1} in BRD area affected by the infiltration of water from Besòs River that contains a large proportion of WWTP effluents. In comparison with other urban GWs, average levels here found were, in general, higher than the ones found in Vulliet and Cren-Olivé (2011) and Strauch et al. (2008), but lower than in Teijon et al. (2010) for the few pharmaceuticals included in those studies. Maximum concentration determined in the present study were mostly higher than the ones reported by Teijon et al. (2010) also in Barcelona, (Grujić et al., 2009) in Serbia and (Standley et al., 2008) in USA. In Table 4 a summary of other studies of determination of pharmaceuticals in urban GWs is shown.

On the other hand, compounds found in rather high concentrations in SWs such as acetaminophen, sulfamethazine and atenolol (López-Serna et al., 2011, 2012) presented low concentrations in the GWs monitored in the present study. This may be due to degradation or adsorption processes that occur during the passage through the soil.

The levels of TPs found cannot be easily compared because of the lack of previous studies of presence of TPs in GW. In fact only the N-acetyl sulphonamides were previously reported in GWs in the Catalonia (Spain) (García-Galán et al., 2011). N-acetyl sulfamethoxazole and N-acetyl sulfamethazine were found at slightly higher mean concentration and frequency than in the present study. Fig. 2 shows the ranges of concentrations quantified for all the TPs. That chart shows that salicylic acid was detected in all samples reaching maximum concentration of 620 ng L^{-1} in one of the wells in BRD zone. For other TPs the concentrations found in Barcelona GWs were lower than the ones reported for SWs, such as carbamazepine TPs

Table 2
Target compounds according to their therapeutic groups and their range (minimum and maximum) and mean concentration in ng L^{-1} found in the groundwater underlying the metropolis of Barcelona (Spain).

Therapeutic group	N°	Compound[a]	Pharmacologic activity	CAS number	MS				PS				BRD			
					Concentration (ng L^{-1})[b]			Freq. (%)[c]	Concentration (ng L^{-1})[b]			Freq. (%)[c]	Concentration (ng L^{-1})[b]			Freq. (%)[c]
					Minimum	Maximum	Average		Minimum	Maximum	Average		Minimum	Maximum	Average	
Analgesics/anti-inflammatories and TPs (14)	1	Acetaminophen	Yes	103-90-2	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	15
	2	Codeine	Yes	76-57-3	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	2.4	0.185	38	**n.d.**	**blq.**	**n.d.**	8
	3	Diclofenac	Yes	15307-86-5	**n.d.**	3.14	0.628	40	**n.d.**	1.17	0.184	54	29.5	380	225	100
	4	**4OH diclofenac**	No	64118-84-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	147	45.9	77
	5	**Diclofenac Acyl-β-D-glucuronide**	No	64118-81-6	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	6	Ibuprofen	Yes	15687-27-1	**n.d.**	988	200	60	**n.d.**	2.12	0.163	46	**n.d.**	379	61	92
	7	Indomethacin	Yes	53-86-1	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	8
	8	Ketoprofen	Yes	22071-15-4	**blq.**	215	81.3	100	**n.d.**	29.5	8.67	92	42.6	152	97.7	100
	9	Mefenamic acid	Yes	61-68-7	**blq.**	64.3	30.8	100	**blq.**	13.5	6.37	100	8	16.4	11.1	100
	10	Naproxen	Yes	22204-53-1	**n.d.**	**blq.**	**n.d.**	40	**n.d.**	**blq.**	**n.d.**	31	**n.d.**	5.59	0.43	8
	11	**Salicylic acid**	Yes	69-72-7	50.2	147	102	100	26.6	98.2	58.9	100	33.4	620	110	100
	12	Phenazone	Yes	60-80-0	**n.d.**	12.4	3.46	60	**n.d.**	2.13	0.164	15	12.5	39.7	24.1	100
	13	Phenylbutazone	Yes	50-33-9	**n.d.**	**blq.**	**n.d.**	40	**n.d.**	**blq.**	**n.d.**	46	**n.d.**	**blq.**	**n.d.**	15
	14	Propyphenazone	Yes	479-92-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	23	70	121	91.7	100
Lipid regulators and TPs (8)	15	Atorvastatin	Yes	134523-00-5	2.19	5.12	3.23	100	**n.d.**	13.5	4.98	92	**n.d.**	15.9	3.18	85
	16	**2OH atorvastatin Acyl-β-D-glucuronide**	No	214217-86-4	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	15	**n.d.**	**blq.**	**n.d.**	15
	17	Bezafibrate	Yes	41859-67-0	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	0.527	0.0749	54	**blq.**	25.8	6.64	100
	18	**Clofibric acid**	Yes	882-09-7	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	7.57	1.36	31
	19	Fenofibrate	Yes	49562-28-9	**blq.**	22.3	11.5	100	**blq.**	74.2	25.1	100	**blq.**	28.7	14.6	100
	20	Gemfibrozil	Yes	25812-30-0	**n.d.**	0.821	0.365	80	**n.d.**	2.37	0.46	62	4.34	751	209	100
	21	Mevastatin	Yes	73573-88-3	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	8	**n.d.**	**n.d.**	**n.d.**	0
	22	Pravastatin	Yes	81093-37-0	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	**blq.**	**n.d.**	23	**n.d.**	12.2	1.85	38
Psychiatric drugs and TPs (12)	23	Carbamazepine	Yes	298-46-4	**blq.**	38.6	10.9	100	**n.d.**	17.8	7.06	92	92.2	136	115	100
	24	**2OH carbamazepine**	No	68011-66-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**blq.**	47.9	27.8	100
	25	**3OH carbamazepine**	No	68011-67-6	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	**blq.**	**n.d.**	8	**blq.**	39.9	18.8	100
	26	**10,11-epoxi carbamazepine**	Yes	36507-30-9	**n.d.**	1.8	0.36	40	**n.d.**	1.42	0.109	15	**n.d.**	8.36	2.91	85
	27	**Acridone**	No	578-95-0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	1.4	0.18	23	**n.d.**	8.2	2.99	77
	28	**Acridin**	No	260-94-6	**n.d.**	5.39	1.08	20	**n.d.**	15.8	2.33	38	**n.d.**	10.7	1.17	23
	29	Diazepam	Yes	439-14-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	1.08	0.0831	23	9.07	35.1	17.6	100
	30	**Desmethyl diazepam**	Yes	1088-11-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	12.9	5.51	77
	31	**Oxazepam glucuronide**	No	6801-81-6	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	8
	32	Fluoxetine	Yes	54910-89-3	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	21	5.31	54	**n.d.**	**n.d.**	**n.d.**	0
	33	Lorazepam	Yes	846-49-1	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	54	15.4	69
	34	Paroxetine	Yes	61869-08-7	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	30.2	6.71	46	**n.d.**	5.17	0.398	15
Antihistaminics and TP (5)	35	Cimetidine	Yes	51481-61-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	20.5	4.3	31
	36	Famotidine	Yes	76824-35-6	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	17.8	2.93	23
	37	Loratadine	Yes	79794-75-5	**n.d.**	**blq.**	**n.d.**	40	**n.d.**	23.7	3.14	38	**n.d.**	**blq.**	**n.d.**	8
	38	Ranitidine	Yes	66357-35-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	4.7	0.362	15	**n.d.**	17.6	1.35	8
	39	**Ranitidine N-oxide**	No	73857-20-2	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
Antibiotics and TPs (31)	40	Azithromycin	Yes	83905-01-5	**n.d.**	30.7	16.1	80	31.5	1620	257	100	5.96	36	15.7	100
	41	Chloramphenicol	Yes	56-75-7	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	42	Chlortetracycline	Yes	57-62-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	34.2	2.63	46	**n.d.**	**blq.**	**n.d.**	23
	43	Ciprofloxacin	Yes	85721-33-1	**n.d.**	51	12.3	80	17.5	443	87.9	100	**n.d.**	113	16.6	92
	44	Clarithromycin	Yes	81103-11-9	1.75	2.87	2.42	100	1.62	5.11	2.6	100	4.2	20.5	10.7	100
	45	Danofloxacin	Yes	112398-08-0	**n.d.**	58.7	11.7	20	**n.d.**	543	105	85	**n.d.**	252	29.8	31
	46	Doxycycline	Yes	564-25-0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	188	19.5	38	**n.d.**	27.6	2.12	23
	47	Enoxacin	Yes	74011-58-8	18.4	69.3	34.4	100	11.8	323	75.2	100	**blq.**	179	23.8	100
	48	Enrofloxacin	Yes	93106-60-6	**blq.**	65.2	19.2	100	12.5	264	74.8	100	**blq.**	172	25.3	100
	49	Erythromycin	Yes	114-07-8	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	19	41.3	28.7	100

(*continued on next page*)

Table 2 (*continued*)

Therapeutic group	N°	Compound[a]	Pharmacologic activity	CAS number	MS				PS				BRD			
					Concentration (ng L^{-1})[b]			Freq. (%)[c]	Concentration (ng L^{-1})[b]			Freq. (%)[c]	Concentration (ng L^{-1})[b]			Freq. (%)[c]
					Minimum	Maximum	Average		Minimum	Maximum	Average		Minimum	Maximum	Average	
	50	**Anhydroerythromycin**	No	23893-13-2	**n.d.**	0.607	0.316	80	**n.d.**	2.26	0.675	54	**n.d.**	8.51	4.2	92
	51	Flumequine	Yes	42835-25-6	**n.d.**	9.2	5.13	80	3.69	10.3	6.26	100	**n.d.**	6.05	4.15	92
	52	Josamycin	Yes	16846-24-5	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	3.8	0.292	8	**n.d.**	**n.d.**	**n.d.**	0
	53	Metronidazole	Yes	443-48-1	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	8
	54	Nifuroxazide	Yes	965-52-6	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	55	Norfloxacin	Yes	70458-96-7	**blq.**	81	33	100	16.6	462	123	100	**n.d.**	308	36.4	69
	56	Ofloxacin	Yes	82419-36-1	10.2	43.3	24.1	100	13.1	367	79.5	100	**blq.**	226	29.6	100
	57	Oxytetracycline	Yes	79-57-2	**n.d.**	12.2	2.44	40	**n.d.**	41	6.52	31	**n.d.**	40.8	4.78	31
	58	Roxithromycin	Yes	80214-83-1	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	3.23	0.745	38	**n.d.**	23.8	7.95	54
	59	Spiramycin	Yes	8025-81-8	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	2980	300	69	**n.d.**	17.2	3.42	23
	60	Sulfadiazine	Yes	68-35-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	37.1	6.4	31	8.23	208	65	100
	61	**N-acetyl sulfadiazine**	No	127-74-2	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	37.2	8.2	46
	62	Sulfamethazine	Yes	57-68-1	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	29.1	4.83	23	14.6	29.2	21.8	100
	63	**N-acetyl sulfamethazine**	No	100-90-3	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	64	Sulfamethoxazole	Yes	723-46-6	**n.d.**	65	22.9	80	**n.d.**	18.2	9.66	85	**blq.**	42.8	13.2	100
	65	**N-acetyl sulfamethoxazole**	No	21312-10-7	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	**blq.**	**n.d.**	15	**n.d.**	**blq.**	**blq.**	62
	66	**Sulfamethoxazole-β-D-glucuronide**	No	14365-52-7	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	67	Tetracycline	Yes	60-54-8	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	141	17.3	54	**n.d.**	56.3	4.33	8
	68	Tilmicosin	Yes	108050-54-0	**n.d.**	5.71	1.14	20	**n.d.**	820	102	92	**n.d.**	14.7	1.49	15
	69	Trimethoprim	Yes	738-70-5	**n.d.**	**blq.**	**n.d.**	20	0.869	9.41	3.04	100	**n.d.**	4.89	0.556	31
	70	Tylosin	Yes	1401-69-0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
Cardiovascular drugs and TPs (16)	71	Atenolol	Yes	29122-68-7	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	8
	72	Betaxolol	Yes	63659-18-7	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	73	Carazolol	Yes	57775-29-8	**n.d.**	1.28	0.62	80	**blq.**	65.5	9	100	**n.d.**	1.75	0.592	54
	74	Enalapril	Yes	75847-73-3	**n.d.**	0.339	0.0678	20	**n.d.**	1.16	0.18	31	**n.d.**	0.322	0.0248	31
	75	**Enalaprilat**	Yes	84680-54-6	2.9	8.5	4.78	100	**n.d.**	12.5	4.75	77	**n.d.**	12.3	2.42	38
	76	Furosemide	Yes	54-31-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	22.8	1.75	8	**blq.**	284	147	100
	77	Hydrochlorothiazide	Yes	58-93-5	1.23	24.6	6.39	100	1.46	4.48	2.54	100	8.62	665	262	100
	78	Lisinopril	Yes	83915-83-7	**n.d.**	8.17	1.63	20	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	46.5	8.04	77
	79	Metoprolol	Yes	37350-58-6	122	176	151	100	95.3	165	135	100	136	355	191	100
	80	Nadolol	Yes	42200-33-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	0.789	0.0607	8	**n.d.**	4.17	0.321	15
	81	Pindolol	Yes	13523-86-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	13.2	1.97	46	**n.d.**	**blq.**	**n.d.**	8
	82	Propranolol	Yes	525-66-6	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	9.38	1.75	23	**n.d.**	**n.d.**	**n.d.**	0
	83	**Propranolol-β-D-glucuronide**	No	NA	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	84	**4OH propranolol**	Yes	62117-35-5	6.11	17.9	10.4	100	**blq.**	21.4	9.72	100	**blq.**	18.6	11.6	100
	85	Sotalol	Yes	3930-20-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	15	**n.d.**	20.1	4.34	77
	86	Timolol	Yes	26839-75-8	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	1.39	0.107	8	**n.d.**	3.88	0.475	23
β-agonists (2)	87	Albuterol	Yes	18559-94-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**blq.**	**n.d.**	15
	88	Clenbuterol	Yes	37148-27-9	**n.d.**	**blq.**	**n.d.**	20	**n.d.**	0.732	0.0563	8	**n.d.**	**blq.**	**n.d.**	15
Barbiturates (3)	89	Butalbital	Yes	77-26-9	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	90	Pentobarbital	Yes	76-74-4	**n.d.**	17.7	3.54	40	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	3.19	0.245	46
	91	Phenobarbital	Yes	50-06-6	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	47.2	9.69	69	**n.d.**	44.4	3.42	8
Antidiabetic and TP (3)	92	Glyburide	Yes	10238-21-8	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	9.25	1.25	54	**blq.**	16.5	7.8	100
	93	**Cis 3OH glyburide**	Yes	23074-02-4	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
	94	**Trans 4OH glyburide**	Yes	23155-00-2	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0	**n.d.**	**n.d.**	**n.d.**	0
Antineoplastic (1)	95	Tamoxifen	Yes	10540-29-1	11.2	39.3	26.9	100	18.9	223	72.7	100	9.25	88.3	35.9	100

n.d.: below limit of detection (non detected).
blq.: below limit of quantification.
[a] In bold the 23 TPs and with no special format, the 72 pharmaceuticals included in the method.
[b] Values below the limit of detection and below the limit of quantification were considered 0 to calculate the average.
[c] Frequency of concentrations above limit of detection.

Table 3
% Absolute and relative method recoveries in HPLC grade water, GW and SW.

Therapeutic group	Compound	% Absolute recoveries			% Relative recoveries		
		HPLC	GW	SW	HPLC	GW	SW
Analgesics/ anti-inflammatories	Acetaminophen	40.27	26.97	97.43	98.74	92.07	145.57
	Codeine	98.87	82.13	60.64	138.14	131.52	129.81
	Diclofenac	60.96	57.84	90.2	100.87	102.48	100.19
	Ibuprofen	63.13	58.22	88.48	96.73	101.98	186.14
	Indomethacin	25.59	26.14	42.46	94.65	99.45	100.9
	Ketoprofen	58.52	117.26	118.79	143.01	155.1	287.84
	Mefenamic acid	68.77	70.88	93.61	97.71	104.61	101.39
	Naproxen	17.98	32.2	83.38	108.04	99.41	115.81
	Salicylic acid	150.97	103.79	142.08	113.92	94.92	93.94
	Phenazone	87.3	74.02	60.08	106.13	102.82	111.65
	Phenylbutazone	78.05	58.08	45.73	95.22	80.51	85.35
	Propyphenazone	68	62.1	63.85	82.56	86.22	118.79
Lipid regulators	Atorvastatin	72.81	72.52	84.15	82.85	77.76	92.99
	Bezafibrate	62.57	84.91	98.22	100.59	95.42	86.5
	Clofibric acid	79.5	83.77	78.96	92.83	94.21	103.52
	Fenofibrate	112.5	104.89	99.95	109.3	106.97	112
	Gemfibrozil	82.98	94.68	77	111.77	131.79	96.27
	Mevastatin	96.37	90.75	85.2	103.59	103.47	95.2
	Pravastatin	99.34	62.88	62.31	68.37	74.53	72.2
Psychiatric drugs	Carbamazepine	64.59	60.71	59.13	110.46	114.53	118.97
	Diazepam	88.93	85.46	82.2	101.5	106.97	104.1
	Fluoxetine	36.07	33.13	22.26	114.67	102.44	98.79
	Lorazepam	116.65	88.76	96.83	132.31	111.79	121.36
	Paroxetine	37.69	39.1	32.41	94.05	103.37	109.66
Antihistaminics	Cimetidine	14.41	17.29	10.16	97.57	90.12	84.23
	Famotidine	29.16	37.63	25.74	122.53	117.7	124.81
	Loratadine	44.64	48.37	37.33	110.88	109.08	102.07
	Ranitidine	28.64	33.03	20.39	194.52	174.14	169.9
Antibiotics	Azithromycin	58.03	84.53	41.62	106.77	97.49	115.6
	Chloramphenicol	87.07	82.52	78.92	97.98	99.17	76.09
	Chlortetracycline	81.12	77.47	99.67	60.62	81.45	60.64
	Ciprofloxacin	89.35	152.12	66.59	105.53	328.33	162.38
	Clarithromycin	30.49	31.93	24.6	81.84	82.21	94.57
	Danofloxacin	53.12	72.42	74.17	72.61	190.4	195.22
	Doxycycline	169.82	171.39	152.68	91.74	131.91	118
	Enoxacin	76.16	112.59	82.02	102.11	266.1	194.8
	Enrofloxacin	61.13	75.82	65.58	92.22	193.58	193
	Erythromycin	121.81	86.79	82.35	67.18	38.83	51.57
	Flumequine	123.04	104.84	103.27	105.27	99.22	98.66
	Josamycin	25.07	29.62	15.53	67.39	75.48	59.71
	Metronidazole	86.98	75.46	71.35	78.78	58.42	72.19
	Nifuroxazide	123.94	122.18	106.53	212.75	230.18	215.77
	Norfloxacin	66.15	136.31	68.33	65.5	243.89	154.3
	Ofloxacin	63.47	83.63	49.05	86.11	200.16	117.34
	Oxytetracycline	147.22	122.79	86.27	78.17	92.94	55.64
	Roxithromycin	43.18	42.89	25.13	116.76	113.24	96.25
	Spiramycin	44.02	66.2	20.11	80.74	76.11	56.73
	Sulfadiazine	91.61	88.91	105.25	116	116.93	165.93
	Sulfamethazine	92.35	87.35	74.44	117.36	115.46	118.17
	Sulfamethoxazole	84.76	87.29	74.92	106.75	119.34	97.29
	Tetracycline	173.97	179.46	141.09	90.21	133.74	94.1
	Tilmicosin	27.53	51	15.17	50.33	58.64	41.76
	Trimethoprim	75.65	70.48	52.39	129.5	132.29	106.75
	Tylosin	33.57	37.06	24.13	90.24	93.25	91.81
Cardiovascular drugs	Atenolol	46	42	30.95	117.43	115.15	120.36
	Betaxolol	56.29	54.53	47.75	102.43	62.87	81.09
	Carazolol	50.44	50.69	42.96	91.77	58.36	120.52
	Enalapril	87.51	83.23	78.43	97.91	97.9	98.12
	Furosemide	74.83	90.26	97.91	99.87	98.71	99.06
	Hydrochlorothiazide	84.39	66.5	66.77	111	106.59	120.62
	Lisinopril	33.03	38.43	37.18	84.73	106.57	146.93
	Metoprolol	75.87	64.56	39.27	194.5	183.84	156.07
	Nadolol	73.76	65.4	36.55	188.64	183.91	146.08
	Pindolol	70.02	62.7	52.38	179.45	177.71	210.33
	Propranolol	53.06	52.23	47.17	96.55	60.14	131.88
	Sotalol	62.6	54.28	38.98	160.53	151.86	156
	Timolol	77.6	63.71	51.06	115.36	115.9	125.42
β-agonists	Albuterol	10.11	8.93	6.34	111.21	115.85	111.98
	Clenbuterol	64.04	61.59	45.69	116.55	70.91	127.84
Barbiturates	Butalbital	77.75	77.7	87.69	98.5	88.34	94.32
	Pentobarbital	95.05	89.91	82.5	113.79	94.02	110.9
	Phenobarbital	98.08	86.57	90.56	98.07	91.12	98.68
Antidiabetic	Glyburide	138.13	122.96	164.65	105.26	102.5	134.22
Antineoplastic (1)	Tamoxifen	10.58	10.36	10.7	131.33	74.16	88.61

Table 4
Presence of pharmaceuticals in other studies of urban GWs.

	Concentration (ng L^{-1})			Reference
	Min	Max	Mean	
Atenolol	18	106	60.8	(Teijon et al., 2010)
Carbamazepine	2	118	39.8	
Codeine	106	348.3	227.15	
Diclofenac	35	477	256	
Furosemide	24	138	91	
Gemfibrozil	12	574	165.3	
Hydrochlorothiazide	2	594	186.54	
Ibuprofen	n.d.	185	185	
Naproxen	145	263	204	
Ofloxacin	4	48	23.01	
Sulfamethazine	6	446	74.75	
Sulfamethoxazole	2	117	47.57	
Carbamazepine	N/A	N/A	20	(Strauch et al., 2008)
Acetaminophen	–	–	–	(Grujić et al., 2009)
Azithromycin	25	140	82.5	
Carbamazepine	6	23	–	
Trimethoprim	100	100	100	
Carbamazepine	<0.5	2.4	N/A	(Standley et al.,
Ibuprofen	<10	19	N/A	2008)
Sulfamethoxazole	0.63	2.2	N/A	
Trimethoprim	1.4	11	N/A	
Acetaminophen	N/A	N/A	10.3	(Vulliet and
Atenolol	N/A	N/A	5.5	Cren-Olivé, 2011)
Bezafibrate	N/A	N/A	0	
Carbamazepine	N/A	N/A	10.4	
Diclofenac	N/A	N/A	9.7	
Ibuprofen	N/A	N/A	0	
Ketoprofen	N/A	N/A	2.8	
Lorazepam	N/A	N/A	1.2	
Metoprolol	N/A	N/A	0.3	
Metronidazole	N/A	N/A	0	
Naproxen	N/A	N/A	1.2	
Ofloxacin	N/A	N/A	0	
Pravastatin	N/A	N/A	0	
Propranolol	N/A	N/A	1.8	
Roxithromycin	N/A	N/A	1.3	
Salycilic acid	N/A	N/A	6.5	
Sulfamethoxazole	N/A	N/A	3	
Trimethoprim	N/A	N/A	1.4	

(10,11-epoxi carbamazepine, 2OH carbamazepine and 3OH carbamazepine) in the SWs in Huerta-Fontela et al. (2011), Langford and Thomas (2011) and López-Serna et al. (2012), ranitidine N-oxide (López-Serna et al., 2012) and for oxazepam glucuronide. In contrast, 4OH diclofenac was found at average concentration of 19.2 ng L^{-1} and maximum concentration of 147 ng L^{-1}) which is the highest concentration reported for environmental waters (Langford and Thomas, 2011; López-Serna et al., 2012).

Relative to the concentrations of parent compounds, the concentrations of TPs were lower than their corresponding parent pharmaceuticals, except for 4OH propranolol and enalaprilat which were 7 and 3 times, respectively, higher than their parent compounds. Carbamazepine TPs were found in concentrations much lower than the parent carbamezepine, while in SW 10,11-epoxi carbamazepine was found at 15, 5 and 4 times higher concentrations than parent carbamazepine (Huerta-Fontela et al., 2011; Langford and Thomas, 2011; López-Serna et al., 2012). Similarly, desmethyl diazepam was found at higher concentration than diazepam in the SWs surveyed by González Alonso et al. (2010), while GWs from BRD area analyzed in the present study show average concentration of 5.5 ng L^{-1} for desmethyl diazepam and 17.6 ng L^{-1} for diazepam.

3.2. Spatial distribution

PSP-2 in PS area turned out being the most contaminated site (see Fig. 3), especially due to the high concentrations of some macrolide antibiotics, as well as fluoroquinolones. In fact, the highest individual concentrations were found in that sampling site, with 2980 ng L^{-1} and 1620 ng L^{-1}, for spiramycin and azithromycin, respectively. PSP-3, the well CAL-2 and PSP-5 also showed total concentration (sum of all analyzed pharmaceuticals) above 1000 ng L^{-1}.

Nevertheless, BRD was the most polluted GW area in Barcelona. Most of the sites in that area (10 out of 13) showed total concentrations above 1000 ng L^{-1}.

In MS under the city centre of Barcelona, only the well GRA-1 presented a total concentration above 1000 ng L^{-1}, and its main contributor was the analgesic ibuprofen with 988 ng L^{-1}.

As it was pointed out previously, GW samples belonging to the BRD area are mainly recharged by natural bank filtration from Besòs River, whose flow contains a high percentage of effluents from WWTPs. However, compounds in SAP-2B and SAP-4 were observed at similar concentrations or even higher than in the river Besòs itself (sample BESOS). Hence, accumulated concentration for all the pharmaceutical-type compounds was slightly higher in SAP-4 than in the river (sample BESOS). Those two sites are situated very near to the edge of the river and at upper depths, but nevertheless, very little attenuation was observed in all the GWs belonging to this area, in general. Almost identical situation was reported for the illicit drugs in Jurado et al. (2012). It should be taken into consideration that the sampling for BESOS was carried out in spring (May), which had especially abundant rainfalls. Therefore the dilution of the contaminants occurred. On the other hand, aquifers work as reservoirs. Thus, GWs under BRD store water belonging to several previous seasons, including dried ones, when the proportion of contaminants was higher. That could be the main reason provoking that situation, since attenuation was claimed by Jurado et al. (2012) to take place in a big extent caused by biologic degradation and/or physical adsorption in this reducing subsoil.

3.3. Profile according to groundwater depth

Every sample in the present monitoring comes from a different sampling site (piezometer or well), but some of them are situated close to each other geographically, but at different depths. Comparable sites are marked in grey shade and separated by discontinuous line in Table 1. Jurado et al., 2012 reported attenuation taking place in all three zones. This should agree with a decrease in the concentrations found in lower sites compared to upper ones. That situation is obvious in BRD zone. In fact, total concentration was clearly higher in SAP-2B (u) than in SAP-1 (m) and SAP-2 (m), as well as in SAP-4 (u) than in SAP-3 (m). See Fig. 3. Since BRD is a reducing area, that depletion was attributed to physical adsorption, biological degradation and/or dilution effect. However, that attenuation in depth was not that obvious in MS and PS, in spite of both areas are oxidizing, so in addition to the attenuation factors previously pointed out, chemical transformation by the components of the soil could also take place. Thus, GRA-2 (l), the deepest site in the whole monitoring, was cleaner than GRA-1 (m) and GRA-3 (u), but on the other hand, GRA-1 (m) with an intermediate depth between GRA-2 (u) and GRA-3 (u), was the most polluted in this area. Similarly, in PS, some pairs like PSP-2/PSP-3 and MPS-1/MSP-2 behaved as expected with decreasing concentrations in depth, but others like PSP-5/PSP-6, PSP-7/PSP-8 and PSP-9/PSP-10 did not. A similar situation was observed for illicit drugs in Jurado et al. (2012). Depths of sewage pipes in every area are unknown to the authors. The possibility of leaking pipes situated deeper than some of the upper sites would explain the present issue, since the sewage water is the main source of pharmaceuticals either in MS and PS, with contribution of 31% and 50%, respectively, versus just 4% in BRD as determined previously by Jurado et al. (2012).

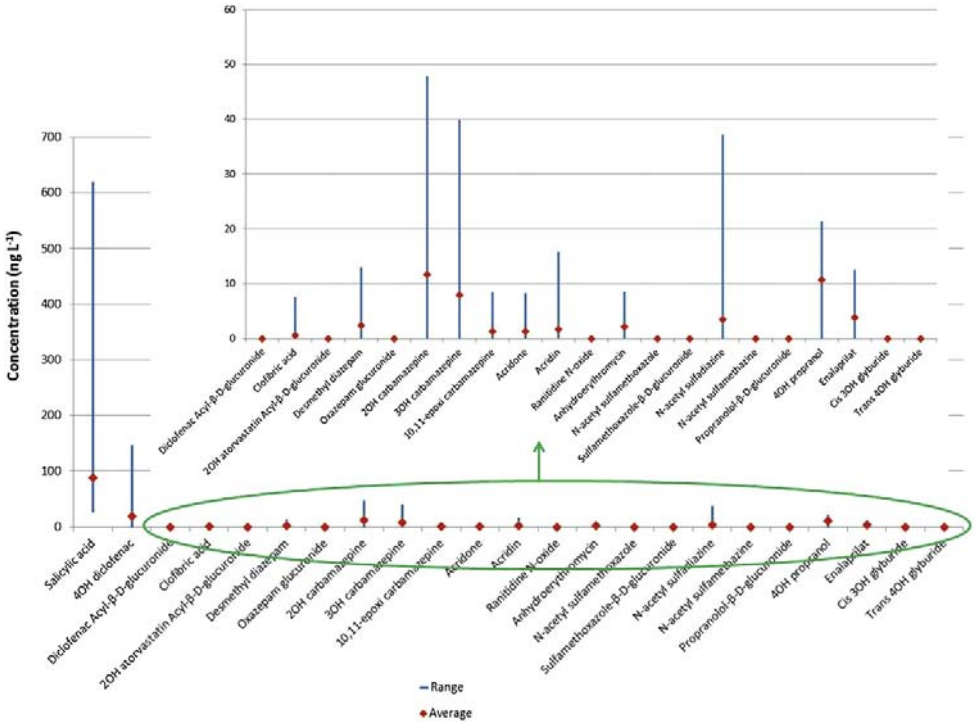

Fig. 2. Range of concentrations for pharmaceuticals TPs in the GWs underlying the metropolis of Barcelona.

3.4. Occurrence according to the geological formation

Table 1 shows the geological composition present in each sampling site. Lithologies consisting of sands and gravels are considered inert to adsorption of the target compounds. On the other hand, the more lipophilic a substance is, the more likely to adsorb onto clays. Fig. 4 shows the average concentration found in sites with geological formation based on both sand and/or gravel, and clay, for a selection of 30 pharmaceuticals and TPs. This selection includes all the ubiquitous and absence compounds, as well as compounds found at high concentrations. They are ordered in descending logarithm of partition coefficient (log *P*), i.e., from

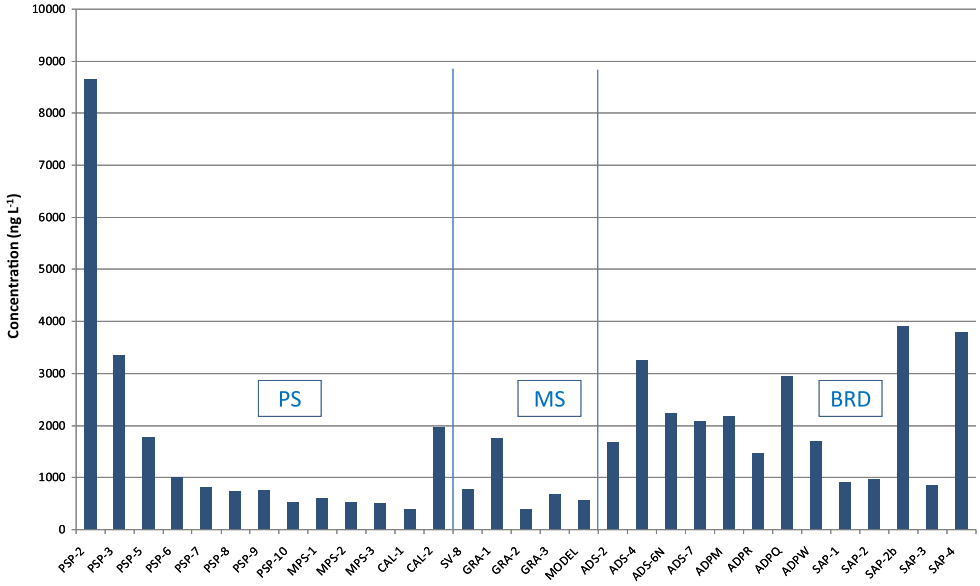

Fig. 3. Pharmaceuticals and TPs total concentration per sampling site.

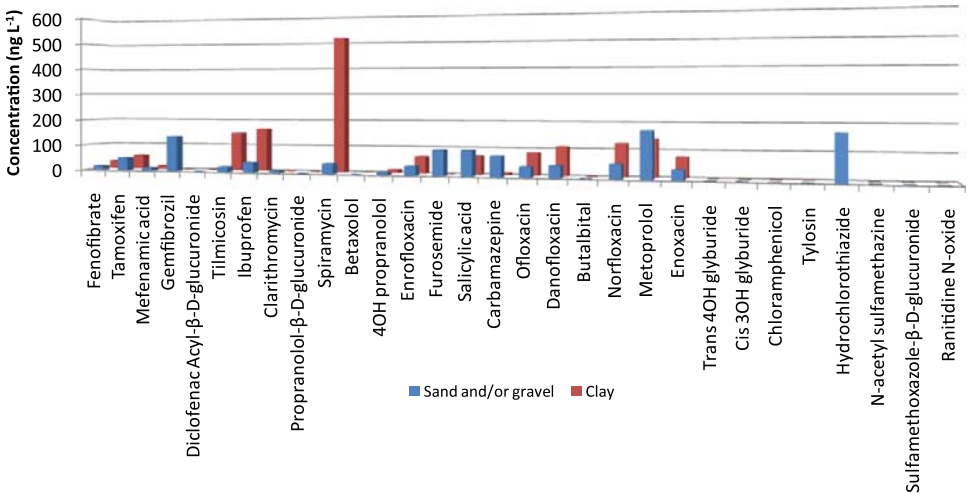

Fig. 4. Average concentration in different geological formations for a selection of pharmaceuticals and TPs.

fenofibrate (log $P = 5.801$) to ranitidine N-oxide (log $P = -3.151$). It might be expected that compounds with high log P partially adsorb onto clays, and therefore, their presence in the surrounding GWs were lower than in aquifers made up sands/gravels. Regarding compounds with low log P, no significant differences may be expected in concentrations between sandy and clay aquifers if geological formation was the only factor influencing. Nonetheless, that situation was not observed here (see Fig. 4). Thus, compounds with high log P like fenofibrate, tamoxifen and mefenamic acid (log $P > 3.8$) presented similar concentrations in both sandy and clay aquifers. Gemfibrozil, with a log P of 4.302, showed higher average concentration in sand/gravel formations, but compounds like tilmicosin and ibuprofen with log P of 3.736 and 3.502, respectively, showed higher average concentration in clay aquifers. On the other hand, compounds with low log P, showed similar average concentration in both types of geological formation, although exceptions like hydrochlorothiazide were also found. In general, a quite random behavior was observed for the levels of concentrations in terms of geological formation, which could mean that lithology is not the main factor influencing in the occurrence of pharmaceuticals and their TPs in the urban GWs.

4. Conclusions

The present work reports for the first time the occurrence of 72 pharmaceuticals along with 23 of their TPs (8 of them still pharmacologically active) in GW of the urban aquifers of the Barcelona metropolitan area (Spain). The main conclusions are as follows:

(1) Concentration levels found were higher than expected for GWs, with maximum concentrations above 100 ng L^{-1} for many compounds especially among analgesics and antibiotics and even higher than 1 μg L^{-1} for two macrolide antibiotics spiramycin and azithromycin. Those are concentrations normally found in SWs, in fact, concentrations found in BRD area were similar or even higher than the ones determined in Besòs River.
(2) Some TPs significantly contributed to the overall levels of pharmaceuticals. For example, salicylic acid was ubiquitous in all monitored areas with concentrations surpassing 600 ng L^{-1}. Furthermore, the active TPs, 4OH propranolol and enalaprilat, were more abundant than their corresponding parent compounds. That should stress the importance of controlling TPs in the environment, especially if they have remaining pharmacological activity.
(3) Aquifers from BRD area, mostly recharged from the Besòs River, whose flow contains a high percentage of effluents from WWTPs, were found to be the most contaminated.
(4) Attenuation of these compounds in depth was observed in BRD zone, but not in PS and MS where pollutants mostly came from leaking sewage pipes.
(5) Geological formation did not show a clear influence in the presence of the target compounds. Percolation source (i.e., losses from sewage system, losses from the water supply network, infiltration from Besòs River, etc), has turned out to be the more important factor influencing in the levels concentration of pharmaceuticals and TPs, followed by depth and geological formation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2012.11.022.

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3.3 Discusión de resultados

Ante la incertidumbre acerca de la existencia y en qué medida de un problema, el primer paso a dar es el desarrollo de herramientas capaces de medir el alcance e intensidad del mismo. Una vez se tienen estas herramientas (en nuestro caso, los métodos de análisis determinístico de fármacos y derivados), éstas se aplican al análisis de escenarios seleccionados por su interés medioambiental y/o socio-económico. Como ya se ha comentado en la introducción de la presente tesis, hace ya unos años que los fármacos estás en el punto de mira como contaminantes medioambientales. A lo largo de este tiempo se han ido perfeccionando las características de estas herramientas, apuntadas en el capítulo anterior como fiabilidad, versatilidad, rendimiento, sensibilidad y capacidad, entre otras. Esta evolución se traduce, por tanto, en la posibilidad de plantear también estudios más avanzados y complejos, y por lo tanto, en ocasiones más útiles, que proporcionen resultados de las mismas características que los métodos. Son varios los escenarios en los que se presenta una necesidad/interés de uso de estas herramientas determinísticas:

a) Conocimiento puntual (espacio-temporal) de las concentraciones de fármacos y sus derivados en un agua medioambiental. Este análisis, se puede englobar dentro de un estudio bi- o tri-dimensional, lo que nos proporcionaría una idea del sistema como un todo, así como relaciones y comparaciones entre los diferentes puntos de muestreo. Ejemplos de esto sería un estudio de las concentraciones en un determinado momento y profundidad de varios puntos a lo largo de una cuenca hidrográfica, o de un sistema de aguas subterráneas. Si en el estudio se incluyen las fuentes de estos analitos, como pueden ser por ejemplo, los efluentes de EDARs, industrias farmacéuticas, etc., se podrá hacer una relación del origen y destino de estos compuestos, su evolución a lo largo de la cuenca, su degradación, dilución, adsorción al material en suspensión, sedimentos o suelo, de sistema hídrico, etc. Si además se incluyen las entradas a las EDARs, el estudio puede dar también una idea del comportamiento de estas sustancias durante el tratamiento en las mismas (degradación, transformación, adsorción a los fangos, etc.). Estudios de este tipo constituyen la primera aproximación que nos da una idea del alcance del problema. Con estos primeros datos, se pueden establecer comparaciones con situaciones similares en otros sistemas hídricos en otras zonas del planeta.

b) Un estudio puntual no es realmente representativo. El sistema puede estar sometido a condiciones meteorológicas, de funcionamiento de las EDARs, etc. por lo que se hacen necesarios estudios similares en el tiempo. Esto, además de darnos idea de la fluctuación natural del sistema, puede mostrar la evolución

en el tiempo del problema medioambiental, evidenciando una tendencia creciente, decreciente o estable.

c) El conocimiento de los niveles de concentración propician estudios de ecotoxicidad a escala de laboratorio, mucho más factibles que a escala real, así como de ecotoxicidad teórica. Estas pruebas junto con un volumen importante de estudios determinísiticos a escala real, presionan sobre las entidades competentes hacia la legislación y regulación de la entrada de este tipo de contaminantes al medioambiente.

d) Con la existencia de legislación o incluso anticipándose a ella, el aumento en el número de estudios determinísticos de control aumentan, así como la búsqueda de medidas preventivas o correctoras para paliar el problema. Durante la elección de las medidas más adecuadas, el planteamiento de estudios de apoyo con uso de herramientas de análisis es imprescindible.

e) Tras la toma de medidas, se hacen necesarios el uso de estudios de seguimiento y control.

La Tabla 7 muestra un resumen de las características principales de los cuatro estudios determinísticos de fármacos y derivados, en aguas medioambientales y residuales incluidos en la presente tesis, que arrojan luz sobre la situación de contaminación hidrológica por estos compuestos en la Península Ibérica.

El Estudio 1 constituye la segunda campaña (escenario b)) del seguimiento y control (escenario e)) de la reutilización del agua residual tras un tratamiento terciario para mantener el caudal ecológico del río Llobregat (Noreste de la Península Ibérica) que además abastece a una ETAP. En este estudio, además de fármacos y derivados, se incluyen drogas de abuso y estrógenos. Comparando los resultados con los de la primera campaña realizada un año antes en el 2008 ([327]), las concentraciones de fármacos y derivados en los mismos puntos de muestreo, fueron en general mayores, delimitando el panorama esperable en situaciones de reutilización. La presencia de fármacos en el río Llobregat ha sido estudiada con anterioridad desde el año 2000 [189, 318, 328, 329]. Los concentraciones encontradas en el Estudio 1, fueron similares o incluso menores a las detectadas en ellos para los compuestos comunes, lo que indica una situación de cierta estabilidad. El Estudio 1 aprovecha, además, los niveles encontrados, para hacer una evaluación de riesgo ecotoxicológico y estrogénico en las zonas muestreadas del río (escenario c)). Este estudio constituye también un escenario e), puesto que evalua la eficiencia del tratamiento terciario de una EDAR en la eliminación de estos compuestos.

El Estudio 2, realizado durante el otoño del 2010, es un claro ejemplo de escenario b). Los niveles de fármacos en el río Ebro han sido estudiados con anterioridad por [75,

205, 330] en los años (2004-2005), (2005-2008), y (2009-2010), respectivamente. Estos estudios, especialmente los dos últimos, son similares en analitos y puntos de muestreo analizados. La tendencia que se ha observado a largo de estos siete años es una estabilidad general en los niveles de concentración encontrados, en cuanto a niveles promedio y máximos. Las diferencias están dentro del rango asumible de variación de método de análisis, en el punto de muestreo, variaciones puntuales metereológicas o hidrológicas, etc. También se mantiene el perfil de puntos más contaminados y limpios, así como la tendencia a encontrar niveles más elevados en afluentes que en el río principal, lo que se atribuye a su menor factor de dilución. En todas los estudios se ha observado, además, un mantenimiento general de los niveles a lo largo del río principal, desde su nacimiento hasta la desembocadura, a pesar de recoger las aguas residuales de varias ciudades de elevada población, lo que se atribuye a que, a pesar de que la carga contaminante de fármacos sea presumiblemente mayor, las concentraciones se mantienen al aumentar el caudal del río también. Por otro lado, el Estudio 2 ha sido pionero en la determinación de gran cantidad de TPs, con todo lo que ello conlleva de comparación con respecto a los niveles de los fármacos de los que derivan, lo que le confiere entidad de escenario a) en ese sentido. Sin embargo, son deseables más trabajos en este sentido en el futuro, que ayuden a establecer rangos de concentración habituales para estos TPs en los diferentes compartimentos medioambientales, y su evolución con el tiempo, así como estudios de ecotoxicidad de los mismos, para poder tomar medidas preventivas o correctoras adecuadas en caso de que fueran necesarias.

El Estudio 3 ha sido pionero en España (escenario a)), y en general, hay muy pocos en el mundo que informen sobre concentraciones de fármacos y derivados, en aguas subterráneas urbanas; mucho menos que incluyan tal cantidad de residuos y TPs. En la publicación científica 7 se comparan los resultados obtenidos con todos ellos. No obstante, estudios periódicos de control serían bienvenidos para afianzar los resultados aquí obtenidos, así como observar evoluciones en su comportamiento con el tiempo. También, estudios adicionales en GWs urbanas bajo otras ciudades españolas como Madrid y alrededores, donde son utilizadas para obtener agua potable, ayudarían a promulgar leyes y regulaciones sobre la entrada de estas sustancias en el medio ambiente.

El Estudio 4 constituye, de nuevo, un escenario a), siendo la primera vez que se realiza una determinación enantiomérica de fármacos quirales en aguas medioambientales españolas. Es incluso la primera referencia de análisis de fármacos en la cuenca del río Guadalquivir, uno de los ríos más importantes de la Península Ibérica, así como de algunas de las EDARs más importantes asociadas. En la publicación científica 4 se discuten y comparan los resultados absolutos obtenidos con los de otros ríos mediterráneos de la Península Ibérica, como el río Ebro y el río Llobregat. En el mundo solo existen otros cuatro estudios enantioméricos de fármacos en el medioambiente,

dos en Canadá, uno en Suecia y otro en Reino Unido. Todos ellos, incluyen aguas residuales, y solo el llevado a cabo en Reino Unido, incluye además aguas superficiales. La comparación de los resultados obtenidos en el Estudio 4 con todos ellos, está recogida en el apartado de *results and discussion* en la publicación científica 4. La repetición de estos estudios, y realización de otros nuevos en otras zonas de interés medioambiental y/o socioeconómico son deseables, así como ensayos de ecotoxicidad enantiomérica, que arrojen luz sobre la gravedad de la situación actual a nivel mundial, para poder así, tomar medidas al respecto si fuera necesario.

Tabla 7. Resumen de las características principales de los cuatro estudios medioambientales incluidos en el capítulo

Estudio	Tipo de escenario	Matriz	Método	Nº compuestos (fármacos + TPs)	Innovación
1	b) c) e)	SW (río Llobregat), EWW	2	74 (72 + 2)	Escenario de reutilización de agua residual. Estudio de ecotoxicidad
2	a) b)	SW (río Ebro)	3	77 (58 + 19)	TPs en el principal río de la Península Ibérica
3	a)	GW urbana (BCN)	2	95 (72 + 23)	Estudio tridimensional de TPs en aguas subterráneas urbanas bajo una gran metrópoli
4	a)	SW (río Guadalquivir), EWW, IWW	4	13 (11 + 2)	Estudio enantiomérico multi-residuo a lo largo de una cuenca hidrográfica

Por otro lado, a continuación se introduce una discusión de presencia de compuestos farmacéuticos a través de los resultados hallados en esta tesis.

Los cuatro estudios aquí presentados tienen en común que el escenario objeto de análisis se encuentra dentro del territorio español, y que la persona que ha llevado a cabo los análisis es la misma en todos los casos, y es la autora de la presente tesis. Por otro lado, estos cuatro estudios presentan diferencias espaciales, temporales y metodológicas, por lo que van aplicaron de manera distinta en cada uno de ellos, los factores que se indicaron en la introducción del presente capítulo. Todo esto introduce dificultad en la comparación de los resultados de los cuatro estudios entre sí, así como de éstos con estudios previos publicados en la bibliografía científica.

En cualquier caso, se pueden encontrar una serie de patrones comunes que parecen aplicar de manera general a las campañas aquí presentadas. La Tabla 8 muestra un resumen de la presencia observada en los cuatro estudios incluidos en esta tesis.

Distribuyendo los hallazgos por matrices, se observa que en las cuatro campañas sobre muestras de SW (una campaña por estudio), para la mayoría de los fármacos analizados, la concentración máxima observada fue inferior a 200 ng L^{-1} y la concentración media no superó los 100 ng L^{-1}. Además, las excepciones a esta regla consistieron habitualmente en alguno o varios de estos tres analgésicos y anti-inflamatorios: diclofenaco, ibuprofeno y acetaminofén; el antibiótico sulfametacina; o la droga cardiovascular hidroclorotiacida. Con respecto a la frecuencia de detección, los analgésicos y anti-inflamatorios ácido salicílico e ibuprofeno, el antibiótico claritromicina, y la droga psiquiátrica carbamacepina, fueron encontrados ubícuos en la mayoría de estas cuatro campañas. Por lo tanto, se podría declarar al ibuprofeno de manera individual, y a los analgésicos y anti-inflamatorios y los antibióticos, en cuanto a clases terapéuticas, como los fármacos más frecuentemente encontrados y a mayores concentraciones en las aguas superficiales de los estudios incluidos en la presente tesis. Esto concuerda claramente con lo que se ha venido observando en los últimos años en estudios en todo el planeta, y cuya revisión bibliográfica ha sido plasmada en la introducción del presente capítulo.

En cuanto a GW, la presente tesis reportó datos solo con el Estudio 3, presentando concentraciones máximas y medias, que en general fueron inferiores a los 200 y 100 ng L^{-1}, respectivamente, coincidiendo con los umbrales observados para las SW. También coincidieron, en gran medida, los compuestos que no se ajustaron a esta regla general, como el diclofenaco, el ibuprofeno y la hidroclorotiacida. Sin embargo, el acetaminofén, se mostró claramente menos abundante y frecuente, lo que se puede atribuir a fenómenos de adsorción y/o eliminación química o biológica durante el filtrado de las aguas subterráneas. La claritromicina y el ácido salicílico fueron encontrados también ubicuos en este estudio, pero no sin embargo la carbamacepina, fármaco comúnmente utilizado como trazador indicador del alcance de contaminación antropogénica en acuíferos.

Tabla 8. Resumen de la presencia de compuestos farmacéuticos observada en los Estudios 1, 2, 3 y 4.

Estudio 1	Concentración máxima (ng L^{-1})		Concentración media (ng L^{-1})		Frecuencia
	Mayoría	Excepciones	Mayoría	Excepciones	Ubucuidad
SW (Llobregat)	<200	200-500: Diclofenaco, Eritromicina, Furosemida, Ibuprofeno, Sulfametacine; 500-100: Acetaminofén, Ácido salicílico; >1000: Metoprolol	<100	100-300: Acetaminofén, Ácido salicílico, Diclofenaco, Eritromicina, Furosemida Ibuprofeno, Metoprolol	Ácido clofíbrico, Ácido salicílico, Acitromicina, Atenolol,Bezafibrato, Carbamacepina, Claritromicina, Codeina, Diclofenaco, Eritromicina, Espiramicina, Fenofibrato, Hidroclorotiacida, Ibuprofeno, Indometacina, Loracepam, Metoprolol, Naproxeno, Ofloxacino, Ranitidina, Sotalol, Sulfametoxazol, Trimetoprim
WWE (con terciario)	<600	600-1000: Ácido salicílico, Diclofenaco, Fenofibrato; >1000: Acitromicina, Eritromcina, Furosemida, Sulfametacina,	<300	300-500: Codeina, Diclofenaco, Sulfametacina 500-1000: Ácido salicílico, Eritromicina >1000: Acitromicina, Furosemida	Ácido clofíbrico, Ácido mefenámico, Ácido salicílico, Acitromicina, Atenolol, Bezafibrato, Carbamacepina, Claritromicina, Codeina, Diacepam, Diclofenaco, Enrofloxacino, Eritromicina, Espiramicina, Fenofibrato, Fluoxetina, Furosemida, Furosemida, Glibenclamida, Hidroclorotiacida, Indometacina, Ibuprofeno, Ketoprofeno, Loracepam, Metoprolol, Metronidazole, Naproxeno, Ofloxacino, Propranolol, Roxitromicina, Salbutamol, Sotalol, Sulfametacina, Sulfametoxazol, Trimetoprim

Estudio 2	**SW (Ebro)**	<200	200-500: Atenolol, Diclofenaco, Hidroclorotiacida; 500-1000: Acetaminofén, Sulfametacina; >1000: 10,11-epoxi-Carbamacepina	<100	100-300: 10,11-epoxi-Carbamacepina, Hidroclorotiacida	Ácido salicílico, Carbamacepina, Claritromicina, Propifenazone, Propranolol, Sulfadiacina, Tamoxifén
Estudio 3	**GW (urbana)**	<200	200-500: Ciprofloxacino, Diclofenaco, Enoxacino, Enrofloxacino, Furosemida, Ketoprofeno, Metoprolol, Norfloxacino, Ofloxacino, Sulfadiacina, Tamoxifen; 500-1000: Ácido salicílico, Danofloxacino, Espiramicina, *Gemfibrocilo*, Hidroclorotiacida, Ibuprofeno, Tilmicosin; >1000: Acitromicina	<100	100-300: Acitromicina, Espiramicina, Hidroclorotiacida, Metoprolol	Ácido mefenámico, Ácido salicílico, Claritromicina, Enoxacino, Enrofloxacino, Fenofibrate, Hidroclorotiacida, Metoprolol, Ofloxacino, Tamoxifén, 4OH propranolol
	SW (Besòs)	<200	200-500: Ibuprofeno, Hidroclorotiacida, Phenobarbital; 500-1000: Gemfibrocilo; >1000: -	-	-	-

Estudio 4					
WWI	WWE	SW (Guadalquivir)			
<600	<600	<200	200-500: Naproxeno; 500-1000: -; >1000: Ibuprofeno	>100	100-300: Naproxeno; 300-500: -; >1000:Ibuprofeno
					Ibuprofeno, Naproxeno
		<600	600-1000: -; >1000: Atenolol, Ibuprofeno, Naproxeno	>300	300-500: - ; 500-1000: Atenolol, Naproxeno ; >1000: Ibuprofeno
					Atenolol, Ibuprofeno, Fluoxetina, Ketoprofeno, Naproxeno, Salbutamol
					300-500: Ketoprofeno ; 500-1000: - ; >1000: Atenolol, Ibuprofeno, Naproxeno
					Atenolol, Ibuprofeno, Fluoxetina, Ketoprofeno, Metoprolol, Naproxeno, Norfluoxetina, Propranolol, Salbutamol

Solo los Estudios 1 y 4 incluyeron análisis de agua residual. De manera general, se puede concluir que en lo que a niveles se refiere, en la mayoría de los casos, las concentraciones máximas se mantuvieron por debajo de los 600 ng L^{-1} y las concentraciones medias no superaron los 300 ng L^{-1}. Muestras de agua residual sin tratar solo se analizaron en esta tesis en el Estudio 4, que presentó similares patrones de frecuencia y compuestos individuales más concentrados, que las muestras de agua residual tratada en el mismo estudio, siendo el ibuprofeno, el naproxeno y el atenolol, los compuestos más frecuentes y a mayores concentraciones. Sin embargo, este patrón no coincidió en demasía con el encontrado en las muestras de agua residual tratada del Estudio 1, donde las concentraciones de estos compuestos se mantuvieron por debajo de los umbrales antes nombrados. Ante esto, y comparando con antecedentes en la literatura científica, se observa que es el patrón del Estudio 1, el que más se asemeja al que habitualmente se describe para aguas residuales, debido a la elevada biodegradabilidad imputada a estos compuestos durante el tratamiento secundario de las EDARs. Esto hace pensar, que el análisis enantiomérico, usado en el Estudio 4, no es un buen estimador de niveles totales de compuestos farmacéuticos en las aguas residuales.

4. CONCLUSIONES GENERALES

De los cuatro métodos desarrollados, el Método 2 (Online SPE-LC-MS/MS) es con el que se consiguió aunar mayor número de las características buscadas. Los otros tres, fueron exitosos solo parcialmente, o en cualquier caso en menor cuantía que el Método 2.

- El Método 1 (Ofline SPE, UHPLC-MS/MS) apuesta claramente por un acortamiento en el tiempo total de análisis, si bien no fue suficiente, en comparación con lo conseguido con el Método 2. No obstante, la reducción en el tiempo de análisis instrumental puede ser considerada suficientemente ventajosa como para aliviar los problemas de disponibilidad de equipos en muchos laboratorios de rutina.

- El Método 3 (Online TurboFlow-LC-MS/MS), por su parte, fracasó estrepitosamente en términos de versatilidad, debido a la no adecuabilidad de la técnica de pre-tratamiento en la extracción y *cleanup* de muestras con matriz medioambiental. El método resultante, es aplicable solo al análisis de GW y SW, y presenta elevados tiempos de análisis por muestra, a pesar de tratarse de un método online. Esto es debido a una mal diseño instrumental del sistema de inyección de muestra, lo que además repercute en un elevado consumo de disolventes, lo que le convierte en un método poco ecológico, y por lo tanto, su uso es incoherente en un análisis medioambiental.

- El Método 4 (Ofline SPE, $LC_{enantiomérica}$-MS/MS) se propone como una herramienta multi-residuo para el análisis enantiomérico de productos farmacéuticos. Sin embargo, se ha observado la posibilidad que los métodos enantiomericos no sean apropiados para determinar concentraciones totales (considerando a todos los enantiómeros conjuntamente).

- El Método 2 (Online SPE-LC-MS/MS), se propone como herramienta medioambiental, puesto que da respuesta de la manera más eficientemente posible y haciendo uso de la tecnología más adecuada disponible en la actualidad. No obstante, actualmente existen otras instrumentaciones muy prometedoras como el Orbitrap, que pueden suponer un avance significativo con respecto al método propuesto.

Con respecto a los estudios medioambientales se puede concluir, que han supuesto todo un éxito en cuanto a la finalidad para lo que fueron planteados, cada uno en su ámbito: primer estudio, estudio de evolución y/o soporte a implantación de medidas correctoras.

- El Estudio 1 ha permitido afianzar los resultados observados en la primera campaña, llevada a cabo el año anterior, proporcionando valiosísima información a la Agencia Catalana del Agua (ACA) en la implantación del

tratamiento terciario de la EDAR del Prat de Llobregat, que disminuiría ostensiblemente la liberación de estos micro-contaminantes emergentes en el medioambiente.

- El Estudio 2 ha confirmado la mayor presencia de los analitos en los afluentes frente a la encontrada en el rio Ebro, incluso en las partes bajas del mismo, cercanas a la desembocadura, situación observada en anteriores campañas. En general, los resultados no permiten hablar de variaciones significativas en los niveles y frecuencias halladas a lo largo de los últimos 10 años. Los TPs, analizados por primera vez en la cuenca, se han encontrado a niveles similares a la de los compuestos padre de los que derivan, lo que da pie a incorporar estos productos de transformación en estudios futuros para observar su evolución en el tiempo.

- El Estudio 3 ha permitido dibujar por primera vez la situación de distribución de la contaminación por productos farmacéuticos en los acuíferos bajo la ciudad de Barcelona, mostrando en las zonas nororientales de la ciudad, una gran variedad de compuestos y a niveles muy elevados. Es decir, una presencia similar a la encontrada en el río Besòs, uno de los ríos con más estrés antropogénico de la Península Ibérica, y que es el principal contribuyente a los acuíferos de la zona.

- El Estudio 4 ha dado también una primera versión del perfil enantiomérico de productos farmacéuticos en la cuenca del río Guadalquivir y de algunas de las EDARs asociadas. En esta primera version se ha observado un mantenimiento en el perfil enantiomérico en los tres tipos de matrices (SW, EWW y IWW), y que no siempre se ha correspondido con el de mezclas racémicas, sino que compuestos como la fluoxetina, el propranol y el albuterol, han presentado un exceso de uno de los enantiómeros frente al otro, invitando a diferenciar estos enantiómeros en futuros estudios, puesto que cada uno de estos isómeros quirales presenta una ecotoxicología individual diferente.

- Finalmente, la presencia, niveles y frecuencia, de los compuestos aquí estudiados, han sido, en general, bastante consistentes, encontrándose patrones comunes de concentraciones máximas, media y ubicuidad, tanto entre los estudios aquí incluidos, como con estudios previos llevados a cabo en diferentes zonas del planeta.

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ANEXOS

A1. Compuestos incluidos en los Métodos 1, 2, 3 y/o 4, junto con sus principales características físico-químicas

Grupo terapéutico	Nº	Compuesto*		¿Actividad farmacológica?	Nº CAS	Fórmula	Masa molecular	¿Quiral?	Estructura[1]	pKa a 25ºC	log P a 25ºC	Surrogate correspondiente
		Fármaco	Producto de transformación									
Analgésicos/anti-inflamatorios y TPs (14)	1	ketoprofen		S	22071-15-4	C16 H14 O3	254,28	S(1)		4.23 ± 0.10	2.911 ± 0.331	Naproxen-d3 Ketoprofen-13C-d3 - rac-Ketoprofen-13C-d3
	2	Ibuprofen		S	15687-27-1	C13 H18 O2	206,28	S(1)		4.41 ± 0.10	3.502 ± 0.227	Ibuprofen-d3 Ibuprofen-d3 - Clenbuterol-d9 E1 / Clenbuterol-d9 E2

Analgésicos/anti-inflamatorios y TPs (14)								
5	Diclofenac	S	15307-86-5	C14 H11 Cl2 N O2	296.15	N(0)		
							4.18 ± 0.10	Diclofenac-d4
							4.548 ± 0.572	Diclofenac-d4
								-
4	Indomethacin	S	53-86-1	C19 H16 Cl N O4	357.79	N(0)		
							3.96 ± 0.30	Ibuprofen-d3
							4.251 ± 0.796	Indomethazine-d4
								Ibuprofen-d3
3	Naproxen	S	22204-53-1	C14 H14 O3	230,26	S(1)		
							4.84 ± 0.30	Naproxen-d3
							2.876 ± 0.239	Naproxen-d3
								rac-Naproxen-d3

Analgésicos/anti-inflamatorios y TPs (14)								
8	Mefenamic acid	S	61-68-7	C15 H15 N O2	241.29	N(0)		3.73 ± 0.36; -1.31 ± 0.50	4.834 ± 0.429	Ibuprofen-d3 Mefenamic acid-d3 Mefenamic acid-d3	-
7	Diclofenac Acyl-β-D-glucuronide	N	64118-81-6	C20 H19 Cl2 N O8	472.27	S(5)		2.67 ± 0.70; -2.83 ± 0.50	3.824 ± 0.636	Phenobarbital-d5	- - -
9	4OH diclofenac	N	64118-84-9	C14 H11 Cl2 N O3	312.15	N(0)		4.17 ± 0.10; -1.27 ± 0.50	4.556 ± 0.488	Carbamazepine-d10 Tamoxifen-13C2	- -

Analgésicos/anti-inflamatorios y TPs (14)								
11	Codeine	S	76-57-3	C18 H21 N O3	299.36	S(5)		13.40 ± 0.20; 8.23 ± 0.40
								1.394 ± 0.550
		-	-					Codeine-d3
		-	-					Codeine-d3
10	Salicylic acid	S	69-72-7	C7 H6 O3	138.12	N(0)		3.01 ± 0.10
								2.011 ± 0.247
		-	-					Salicylic acid-d6
								Salicylic acid-d6
								Hydrochlorothiazide-d2
6	Acetaminophen	S	103-90-2	C8 H9 N O2	151.16	N(0)		9.86 ± 0.13; 1.72 ± 0.50
								0.475 ± 0.210
		-	-					Acetaminophen-d4
								Acetaminophen-d4
								Acetaminophen-d4

Analgésicos/anti-inflamatorios y TPs (14)									
14	Phenazone	S	60-80-0	C11 H12 N2 O	188.23	N(0)		0.65 ± 0.65	Phenazone-d3
								0.441 ± 0.255	Phenazone-d3
								-	Phenazone-d3
13	Phenylbutazone	S	50-33-9	C19 H20 N2 O2	308.37	N(0)		4.64 ± 0.10; -0.29 ± 0.40	Phenazone-d3
								3.376 ± 0.263	Carbamazepine-d10
								-	Phenazone-d3
12	Propyphenazone	S	479-92-5	C14 H18 N2 O	230.31	N(0)		1.46 ± 0.65	Phenazone-d3
								1.719 ± 0.260	Carbamazepine-d10
								-	Paroxetine-d4

Reguladores de lípidos y TPs (8)										
17	Fenofibrate	S	49562-28-9	C20 H21 Cl O4	360.83	N(0)		-	5.801 ± 0.388	Fenofibrate-d6
								-		Fenofibrate-d6
								-		Fenofibrate-d6
16	Bezafibrate	S	41859-67-0	C19 H20 Cl N O4	361.82	N(0)		3.29 ± 0.10; -2.06 ± 0.70	2.504 ± 0.419	Bezafibrate-d4
										Bezafibrate-d4
								-		Clofibric acid-d4
15	Clofibric acid	S	882-09-7	C10 H11 Cl O3	214.65	N(0)		3.18 ± 0.10	2.425 ± 0.273	Clofibric acid-d4
										Clofibric acid-d4

Reguladores de lípidos y TPs (8)									
20	Atorvastatin	S	134523-00-5	C33 H35 F N2 O5	558.64	S(2)		4.29 ± 0.10; 0.38 ± 0.50	3.846 ± 0.731	Carbamazepine-d10	Atorvastatin-d5	Atorvastatin-d5	-
19	Pravastatin	S	81093-37-0	C23 H36 O7	424.53	S(8)		4.31 ± 0.10	2.210 ± 0.534	Naproxen-d3	Pravastatin-d3	Pravastatin-d3	-
18	Gemfibrozil	S	25812-30-0	C15 H22 O3	250.33	N(0)		4.75 ± 0.45	4.302 ± 0.324	Ibuprofen-d3	Gemfibrozil-d6	Gemfibrozil-d6	-

Reguladores de lípidos y TPs (8)								
21	2OH atorvastatin Acyl-β-D-glucuronide	N	214217-86-4	C33 H35 F N2 O6	574.64	S(7)		4.29 ± 0.10; 1.86 ± 0.50	4.103 ± 0.747	Flumequine-13C3	Ibuprofen-d3	-
22	Mevastatin	S	73573-88-3	C23 H34 O5	390.51	S(17)		13.49 ± 0.40	3.777 ± 0.480	-	Carbamazepine-d10	Carbamazepine-d10
23	Paroxetine	S	61869-08-7	C19 H20 F N O3	329.37	N(0)		9.68 ± 0.10	3.701 ± 0.436	Paroxetine-d4	Paroxetine-d4	Carbamazepine-d10

Drogas psiquiátricas y TPs (13)								
24	Fluoxetine	S	54910-89-3	C17 H18 F3 N O	309,33	S(1)	10.05 ± 0.10	3.930 ± 0.434
								Fluoxetine-d5 (S+) / Fluoxetine-d5 (R-)
								Fluoxetine-d5
								Fluoxetine-d5
								Fluoxetine-d5
25	Norfluoxetine	S	83891-03-6	C16 H16 F3 N O	295,3	S(1)	9.05 ± 0.13	3.763 ± 0.415
								Fluoxetine-d5 (S+) / Fluoxetine-d5 (R-)
								-
								-
								-
26	Diazepam	S	439-14-5	C16 H13 Cl N2 O	284,74	N(0)	3.40 ± 0.10	2.801 ± 0.694
								Diazepam-d5
								Diazepam-d5
								Diazepam-d5
								-

Drogas psiquiátricas y TPs (13)										
27	Desmethyl diazepam	S	1088-11-5	C15 H11 Cl N2 O	270.71	N(0)		11.72 ± 0.70; 3.22 ± 0.10	2.776 ± 0.802	Ofloxacin-d8	Diazepam-d5	-
28	Oxazepam glucuronide	N	6801-81-6	C21 H19 Cl N2 O8	462.84	S(9)		2.77 ± 0.70	2.430 ± 0.999	Diclofenac-d4	Clofibric acid-d4	-
29	Lorazepam	S	846-49-1	C15 H10 Cl2 N2 O2	321.16	N(0)		10.80 ± 0.70; 0.17 ± 0.50	2.382 ± 0.915	Diazepam-d5	Diazepam-d5	-

Drogas psiquiátricas y TPs (13)									
30	Carbamazepine	S	298-46-4	C15 H12 N2 O	236.27	N(0)		13.94 ± 0.20; -0.49 ± 0.20	1.895 ± 0.597
								Carbamazepine-d10	Carbamazepine-d10
								Carbamazepine-d10	Carbamazepine-d10
								-	-
31	2OH carbamazepine	N	68011-66-5	C15 H12 N2 O2	252.27	N(0)		10.28 ± 0.20; -0.50 ± 0.20	0.481 ± 1.111
								Carbamazepine-d10	Carbamazepine-d10
								-	-
32	3OH carbamazepine	N	68011-67-6	C15 H12 N2 O2	252.27	N(0)		10.00±0.20; -0.47±0.20	1.780 ± 0.603
								Carbamazepine-d10	Phenazone-d3
								-	-

Drogas psiquiátricas y TPs (13)								
33	10,11-epoxi carbamazepine	S	36507-30-9	C15 H12 N2 O2	252.27	S(2)		13.91 ± 0.20; -0.50 ± 0.20
								0.155 ± 0.718
							Carbamazepine-d10	Carbamazepine-d10
							-	-
34	Acridone	N	578-95-0	C13 H9 N O	195.22	N(0)		-0.30 ± 0.30
								3.234 ± 0.730
							Carbamazepine-d10	Carbamazepine-d10
							-	-
35	Acridin	N	260-94-6	C13 H9 N	179,22	N(0)		5.56 ± 0.10
								3.450 ± 0.252
							Carbamazepine-d10	Carbamazepine-d10
							-	-

Antihistamínicos y TP (5)								
38	Ranitidine	S	66357-35-5	C13 H22 N4 O3 S	314,4	N(0)	8.35 ± 0.28	Ranitidine-d6
							-0.068 ± 0.615	Ranitidine-d6
								-
37	Famotidine	S	76824-35-6	C8 H15 N7 O2 S3	337,45	N(0)	7.51 ± 0.40; 8.53 ± 0.70	Cimetidine-d3
							-1.655 ± 0.646	Famotidine-13C3
								Famotidine-13C3
								-
36	Loratadine	S	79794-75-5	C22 H23 Cl N2 O2	382,88	N(0)	4.27 ± 0.20	Cimetidine-d3
							3.895 ± 0.701	Loratadine-d4
								Loratadine-d4
								-

Antibióticos y TPs (31)									
41	Tetracycline	S	60-54-8	C22 H24 N2 O8	444,43	S(3)		4.50 ± 1.00; 11.02 ± 0.70	0.617 ± 0.757
								Carbamazepine-d10	-
								Demeclocycline	-
Antihistamínicos y TP (5)									
40	Cimetidine	S	51481-61-9	C10 H16 N6 S	252,34	N(0)		14.13 ± 0.10; 7.07 ± 0.61	-0.065 ± 0.495
								Cimetidine-d3	-
								Cimetidine-d3	-
								Cimetidine-d3	-
39	Ranitidine N-oxide	N	73857-20-2	C13 H22 N4 O4 S	330,4	N(0)		4.46 ± 0.40	-3.151 ± 0.864
								Ranitidine-d6	-
								Ranitidine-d6	-

Antibióticos y TPs (31)										
44	Clortetraciclina	S	57-62-5	C22 H23 Cl N2 O8	478,88	S(5)		4.50 ± 1.00; 11.01 ± 0.70	1.323 ± 0.767	Carbamazepine-d10	-	-	Demeclocycline
43	Doxyciclina	S	564-25-0	C22 H24 N2 O8	444,43	S(9)		4.50 ± 1.00; 10.84 ± 0.70	1.777 ± 0.760	Carbamazepine-d10	-	-	Demeclocycline
42	Oxytetraciclina	S	79-57-2	C22 H24 N2 O9	460,43	S(9)		4.50 ± 1.00; 10.80 ± 0.70	0.479 ± 0.784	Carbamazepine-d10	-	-	Demeclocycline

Antibióticos y TPs (31)									
45	Eritromycin	S	114-07-8	C37 H67 N O13	733,93	S(18)		13.09 ± 0.70; 8.16 ± 0.70	1.909 ± 0.841
									Erythromycin-13C-d3
									Erythromycin-13C-d3
								-	-
46	Anhydroerythromycin	N	23893-13-2	C37 H65 N O12	715,91	S(16)		13.46 ± 0.70; 8.14 ± 0.70	4.196 ± 0.858
									Erythromycin-13C-d3
									Atorvastatin-d5
								-	-
47	Azithromycin	S	83905-01-5	C38 H72 N2 O12	748,98	S(17)		13.28 ± 0.70; 8.59 ± 0.70	2.582 ± 0.853
									Azithromycin-d3
									Azithromycin-d3
								-	-

Antibióticos y TPs (31)								
50	Josamycin	S	16846-24-5	C42 H69 N O15	827,99	S(15)		13.06 ± 0.70; 7.40 ± 0.70	4.734 ± 0.840	Azithromycin-d3 Clarithromycin-d3 Loratadine-d4 -
49	Claritthromycin	S	81103-11-9	C38 H69 N O13	747,95	S(18)		13.08 ± 0.70; 8.16 ± 0.70	2.805 ± 0.845	Azithromycin-d3 Clarithromycin-d3 Clarithromycin-d3 -
48	Roxithromycin	S	80214-83-1	C41 H76 N2 O15	837,05	S(18)		13.00 ± 0.70; 8.16 ± 0.70	2.842 ± 0.869	Azithromycin-d3 Clarithromycin-d3 Loratadine-d4 -

Antibióticos y TPs (31)								
53	Tylosin	S	1401-69-0	C46 H77 N O17	916,1	S(21)		13.06 ± 0.70; 7.39 ± 0.70	0.628 ± 0.886	Azithromycin-d3	Clarithromycin-d3	-	-
52	Tilmicosin	S	108050-54-0	C46 H80 N2 O13	869,13	S(19)		13.16 ± 0.70; 9.58 ± 0.10	3.736 ± 0.859	Azithromycin-d3	Clarithromycin-d3	-	-
51	Spiramycin	S	24916-50-5	C43 H74 N2 O14	843.05	S(19)		13.06 ± 0.70; 8.61 ± 0.60	2.745 ± 0.879	Azithromycin-d3	Spiramycin-d3	-	-

Antibióticos y TPs (31)									
54	Sulfamethoxazole	S	723-46-6	C10 H11 N3 O3 S	253,28	N(0)		5.81 ± 0.50; 1.39 ± 0.10	0.659 ± 0.409
								Sulfamethoxazole-d4	Sulfamethoxazole-d4
								-	Sulfamethoxazole-d4
55	N-acetyl sulfamethoxazole	N	21312-10-7	C12 H13 N3 O4 S	295,31	N(0)		5.60 ± 0.50; -3.38 ± 0.50	0.955 ± 0.416
								Sulfamethoxazole-d4	Glyburide-d3
								-	-
56	Sulfamethoxazole-β-D-glucuronide	N	14365-52-7	C16 H19 N3 O9 S	429,4	S(5)		2.70 ± 0.70; 0.44 ± 0.10	-2.018 ± 0.571
								-	Salicylic acid-d6
								-	-

Antibióticos y TPs (31)								
57	Sulfadiazine	S	68-35-9	C10 H10 N4 O2 S	250,28	N(0)		6.81 ± 0.10; 1.64 ± 0.10
								-0.074 ± 0.255
								Sulfamethoxazole-d4
								Sulfadiazine-d4
								Sulfadiazine-d4
								-
58	N-acetyl sulfadiazine	N	127-74-2	C12 H12 N4 O3 S	292,31	N(0)		6.08 ± 0.10; -0.22 ± 0.10
								-0.774 ± 0.255
								Ciprofloxacin-d8
								Carbamazepine-d10
								-
59	Sulfamethazine	S	57-68-1	C12 H14 N4 O2 S	278,33	N(0)		7.89 ± 0.10; 1.69 ± 0.10
								0.296 ± 0.278
								Sulfamethoxazole-d4
								Sulfamethazine-d4
								Sulfamethazine-d4
								-

Antibióticos y TPs (31)								
62	Ciprofloxacin	S	85721-33-1	C17 H18 F N3 O3	331,34	N(0)	6.43 ± 0.41; 8.68 ± 0.10	1.625 ± 0.831	Ofloxacin-d8 Ciprofloxacin-d8 -
61	Ofloxacin	S	82419-36-1	C18 H20 F N3 O4	361,37	S(1)	5.19 ± 0.40; 7.37 ± 0.42	1.855 ± 0.875	Ofloxacin-d8 Ofloxacin-d8 Ofloxacin-d8 rac-Ofloxacin-d8
60	N-acetyl sulfamethazine	N	100-90-3	C14 H16 N4 O3 S	320,37	N(0)	7.16 ± 0.10; 1.10 ± 0.10	-0.404 ± 0.277	- Sulfamethazine-d4 Carbamazepine-d10 -

Antibióticos y TPs (31)									
63	Norfloxacin	S	70458-96-7	C16 H18 F N3 O3	319,33	N(0)		0.16 ± 0.20; 8.68 ± 0.10	1.744 ± 0.831	Ofloxacin-d8 Norfloxacin-d5 Norfloxacin-d5 -
64	Danofloxacin	S	112398-08-0	C19 H20 F N3 O3	357,38	S(2)		6.43 ± 0.41; 9.00 ± 0.20	1.811 ± 0.909	Ofloxacin-d8 Ofloxacin-d8 Ofloxacin-d8 -
65	Enoxacin	S	74011-58-8	C15 H17 F N4 O3	320,32	N(0)		6.04 ± 0.70; 8.19 ± 0.10	1.589 ± 0.955	Ofloxacin-d8 Ofloxacin-d8 Glyburide-d3 -

Antibióticos y TPs (31)									
89	Trimethoprim	S	738-70-5	C14 H18 N4 O3	290,32	N(0)		7.04 ± 0.10	Carbamazepine-d10
								0.594 ± 0.385	Trimethoprim-d3
									Trimethoprim-d3
									-
67	Flumequine	S	42835-25-6	C14 H12 F N O3	261,25	S(1)		5.70 ± 0.40;	rac-Flumequine-13C3
								-1.98 ± 0.60	Flumequine-13C3
								0.882 ± 0.669	Flumequine-13C3
									Carbamazepine-d10
99	Enrofloxacin	S	93106-60-6	C19 H22 F N3 O3	359,39	N(0)		6.43 ± 0.41; 7.76 ± 0.10	Ofloxacin-d8
								2.306 ± 0.819	Enrofloxacin-d5
								-	-

Antibióticos y TPs (31)								
71	Metronidazole	S	443-48-1	C6 H9 N3 O3	171,15	N(0)		14.44 ± 0.10; 2.58 ± 0.34
							Metronidazole-d4	-0.135 ± 0.301
							Metronidazole-d4	-
							Ranitidine-d6	
70	Nifuroxazide	S	965-52-6	C12 H9 N3 O5	275,22	N(0)		8.36 ± 0.15; -1.69 ± 0.70
							Carbamazepine-d10	1.824 ± 0.339
							Carbamazepine-d10	-
							-	-
69	Chloramphenicol	S	56-75-7	C11 H12 Cl2 N2 O5	323,13	S(2)		11.03 ± 0.46; -1.73 ± 0.70
							Ibuprofen-d3	1.103 ± 0.354
							Diazepam-d5	-
							Ibuprofen-d3	

Drogas cardiovasculares y TPs (16)							
74	Metoprolol	S	37350-58-6	C15 H25 N O3	267,36	S(1)	
							13.89 ± 0.20; 9.43 ± 0.10
							1.632 ± 0.263
							Atenolol-d7
							Metoprolol-d7
							Metoprolol-d7
							Metoprolol-d7 E1 / Metoprolol-d7 E2
73	Sotalol	S	3930-20-9	C12 H20 N2 O3 S	272,36	S(1)	
							8.28 ± 0.10; 9.31 ± 0.10
							0.240 ± 0.369
							Atenolol-d7
							Sotalol-d6
							Sotalol-d6
							Sotalol-d7 E1 / Sotalol-d7 E2
72	Atenolol	S	29122-68-7	C14 H22 N2 O3	266,34	S(1)	
							13.88 ± 0.20; 9.43 ± 0.10
							0.335 ± 0.279
							Atenolol-d7
							Atenolol-d7
							Atenolol-d7
							Atenolol-d7 (S-) / Atenolol-d7 (R+)

Drogas cardiovasculares y TPs (16)							
77	4OH propranolol	S	62117-35-5	C16 H21 N O3	275,34	S(1)	
							10.17 ± 0.40; 9.60 ± 0.30
	-	-	-	-	-	-	-
	Propranolol-d7						2.450 ± 0.254
	Propranolol-d7 (S-) / Propranolol-d7 (R+)						
76	Propranolol-β-D-glucuronide	N	NA	C22H29NO8	435,47	S(9)	
							2.78 ± 0.70; 9.30 ± 0.30
	-	-	-	-	-	-	-
	Naproxen-d3						2.781 ± 0.713
	Flumequine-13C3						
75	Propranolol	S	525-66-6	C16 H21 N O2	259,34	S(1)	
							13.84 ± 0.20; 9.50 ± 0.30
	Propranolol-d7						2.900 ± 0.247
	Propranolol-d7 (S-) / Propranolol-d7 (R+)						
	Atenolol-d7						

Drogas cardiovasculares y TPs (16)							
80	Carazolol	S	57775-29-8	C18 H22 N2 O2	298,38	S(1)	13.94 ± 0.20; 9.54 ± 0.30
							3.626 ± 0.412
							Atenolol-d7
							Pindolol-d7
							Propranolol-d7
							Metoprolol-d7 E1 / Metoprolol-d7 E2
79	Betaxolol	S	63659-18-7	C18 H29 N O3	307,43	S(1)	13.89 ± 0.20; 9.17 ± 0.38
							2.687 ± 0.401
							Atenolol-d7
							Pindolol-d7
							Propranolol-d7
							Pindolol-d7 E1 / Pindolol-d7 E2
78	Timolol	S	26839-75-8	C13 H24 N4 O3 S	316,42	S(1)	13.38 ± 0.20; 9.35 ± 0.10
							1.280 ± 0.408
							Atenolol-d7
							Timolol-d5
							Timolol-d5
							Timolol-d5 (S-) / Timolol-d5 (R+)

Drogas cardiovasculares y TPs (16)						
83	Enalapril	S	75847-73-3	C20 H28 N2 O5	376,45	S(3)
					3.247 ± 0.738	
					3.15 ± 0.20; 5.43 ± 0.39	
82	Nadolol	S	42200-33-9	C17 H27 N O4	309,4	S(3)
					0.564 ± 0.373	
					13.91 ± 0.20; 9.54 ± 0.10	
81	Pindolol	S	13523-86-9	C14 H20 N2 O2	248,32	S(1)
					1.680 ± 0.250	
					13.94 ± 0.20; 9.54 ± 0.30	

-	Enalapril-d5	Enalapril
-	Enalapril-d5	Enalapril
-	Enalapril-d5	Enalapril
-	Atenolol-d7	Atenolol
-	Pindolol-d7	Pindolol
Pindolol-d7 E1 / Pindolol-d7 E2	Pindolol-d7	Pindolol
-	Atenolol-d7	Atenolol

Drogas cardiovasculares y TPs (16)								
98	Lisinopril	S	76547-98-3	C21 H31 N3 O5	405,48	S(3)		2.18 ± 0.10; 10.50 ± 0.10	3.474 ± 0.746	Atenolol-d7	Atenolol-d7	Atenolol-d7	-
85	Hydrochlorothiazide	S	58-93-5	C7 H8 Cl N3 O4 S2	297,74	N(0)		8.95 ± 0.20; -4.08 ± 0.20	-0.021 ± 0.260	Hydrochlorothiazide-d2	Hydrochlorothiazide-d2	Hydrochlorothiazide-d2	-
84	Enalaprilat	S	76420-72-9	C18 H24 N2 O5	348,39	S(3)		2.20 ± 0.10; 7.79 ± 0.40	3.634 ± 0.745	Enalapril-d5	Enalapril-d5	-

β-agonistas (2)	68	Clenbuterol	S	37148-27-9	C12 H18 Cl2 N2 O	277,19	S(1)	13.29 ± 0.20; 9.51 ± 0.10	2.634 ± 0.384	Clenbuterol-d9 E1 / Clenbuterol-d9 E2	Atenolol-d7
										Clenbuterol-d9	Clenbuterol-d9
	88	Albuterol	S	18559-94-9	C13 H21 N O3	239,31	S(1)	9.99 ± 0.31; 9.62 ± 0.10	0.692 ± 0.379	Albuterol-d3	Albuterol-d3
										Albuterol-d3 E1 / Albuterol-d3 E2	Albuterol-d3
Drogas cardiovasculares y TPs (16)	87	Furosemide	S	54-31-9	C12 H11 Cl N2 O5 S	330,74	N(0)	3.04 ± 0.36; -2.49 ± 0.50	2.304 ± 0.475	Furosemide-d5	Furosemide-d5
										-	Ibuprofen-d3

Barbitúricos (3)								
92	Phenobarbital	S	50-06-6	C12 H12 N2 O3	232,24	N(0)		7.58 ± 0.10	0.529 ± 0.262	Phenobarbital-d5	Phenobarbital-d5	Phenobarbital-d5	-
91	Pentobarbital	S	76-74-4	C11 H18 N2 O3	226,27	N(0)		7.93 ± 0.10	2.176 ± 0.245	Phenobarbital-d5	Phenobarbital-d5	Diclofenac-d4	-
90	Butalbital	S	77-26-9	C11 H16 N2 O3	224,26	N(0)		7.78 ± 0.10	1.790 ± 0.259	Phenobarbital-d5	Phenobarbital-d5	Clofibric acid-d4	-

Antidiabéticos y TP (3)									
95	Trans 4OH glyburide	S	23155-00-2	C23 H28 Cl N3 O6 S	510	S(2)		5.08 ± 0.10; -1.86 ± 0.70	1.518 ± 0.483	Glyburide-d3	-
96	Cis 3OH glyburide	S	23074-02-4	C23 H28 Cl N3 O6 S	510	S(2)		5.08 ± 0.10; -1.86 ± 0.70	1.478 ± 0.483	Glyburide-d3	-
93	Glyburide	S	10238-21-8	C23 H28 Cl N3 O5 S	494	N(0)		5.11 ± 0.10	3.076 ± 0.475	Glyburide-d3	-

Antineoplásticos (1)								
96	Tamoxifen	S	10540-29-1	C26 H29 N O	371,51	N(0)		8.69 ± 0.28	5.133 ± 0.314	Tamoxifen-13C2	Tamoxifen-13C2	-	Carbamazepine-d10

A2. Lista de abreviaturas

ACN: Acetonitrilo

APCI: *Atmospheric Pressure Chemical Ionization*

APPI: *Atmospheric Pressure Photoionization*

AOP: *Advanced Oxidation Process*

CAS: *Conventional Activated Sludge*

cLC: *Capillary-column-switching Liquid Chromatography*

DBO: Demanda Biológica de Oxígeno

DDD: Dosis Diaria Definida

DQO: Demanda Química de Oxígeno

DW: *Drinking Water*

ECD: *Electron Capture Detector*

EDAR: Estación Depuradora de Aguas Residuales

EEUU: Estados Unidos

EMEA: European Medicines Agency

ESI: *ElectroSpray Ionization*

ESI+: *ElectroSpray Ionization* en modo positivo

ESI-: *ElectroSpray Ionization* en modo negativo

ETAP: Estación de Tratamiento de Agua Potable

EWW: *Effluent Waste Water*

FDA: Food and Drug Administration

FID: *Flame Ionization Detector*

GC: *Gas Chromatography*

GW: *Groundwater*

HILIC: *Hydrophilic Interaction Liquid Chromatography*

HPLC: High Pressure/Performance Liquid Chromatography

IWW: *Influent Waste Water*

LC: *Liquid chromatography*

LOD: *Limit of Detection*

LOQ: *Limit of Quantification*

LLME: *Holow Fibre Liquid-Liquid Membrane Extraction*

MeOH: Metanol

MBR: *Membrane Biological Reactor*

MIC: *Minimum Inhibitory Concentration*

MIP: *Molecular Imprinted Polimers*

MS/MS: Espectrometría de Masas en tándem

MS: *Mass Spectrometry*

NI: *Negative Ionization*

PCB: Policlorobifenilo

PET: Polyethylene Terephthalate

PI: *Positive Ionization*

PTFE: PoliTetraFluoroEtileno

Q: Cuadrupolo

QqQ: Triple cuadrupolo

REACH: *Registration, Evaluation, Authorisation and Restriction of Chemicals*

SLME: *Supported Liquid Membrane Extraction*

SPE: *Solid Phase Extraction*

SPME: *Solid Phase MicroExtraction*

SRM: *Selected Reaction Monitoring*

SW: *Superficial Water*

TOF: *Time of Flight*

UHPLC: *Ultra High Pressure/Performance Liquid Chromatography*

USGS: United States Geological Survey

WW: *Waste Water*

A3. Lista de Tablas y Figuras