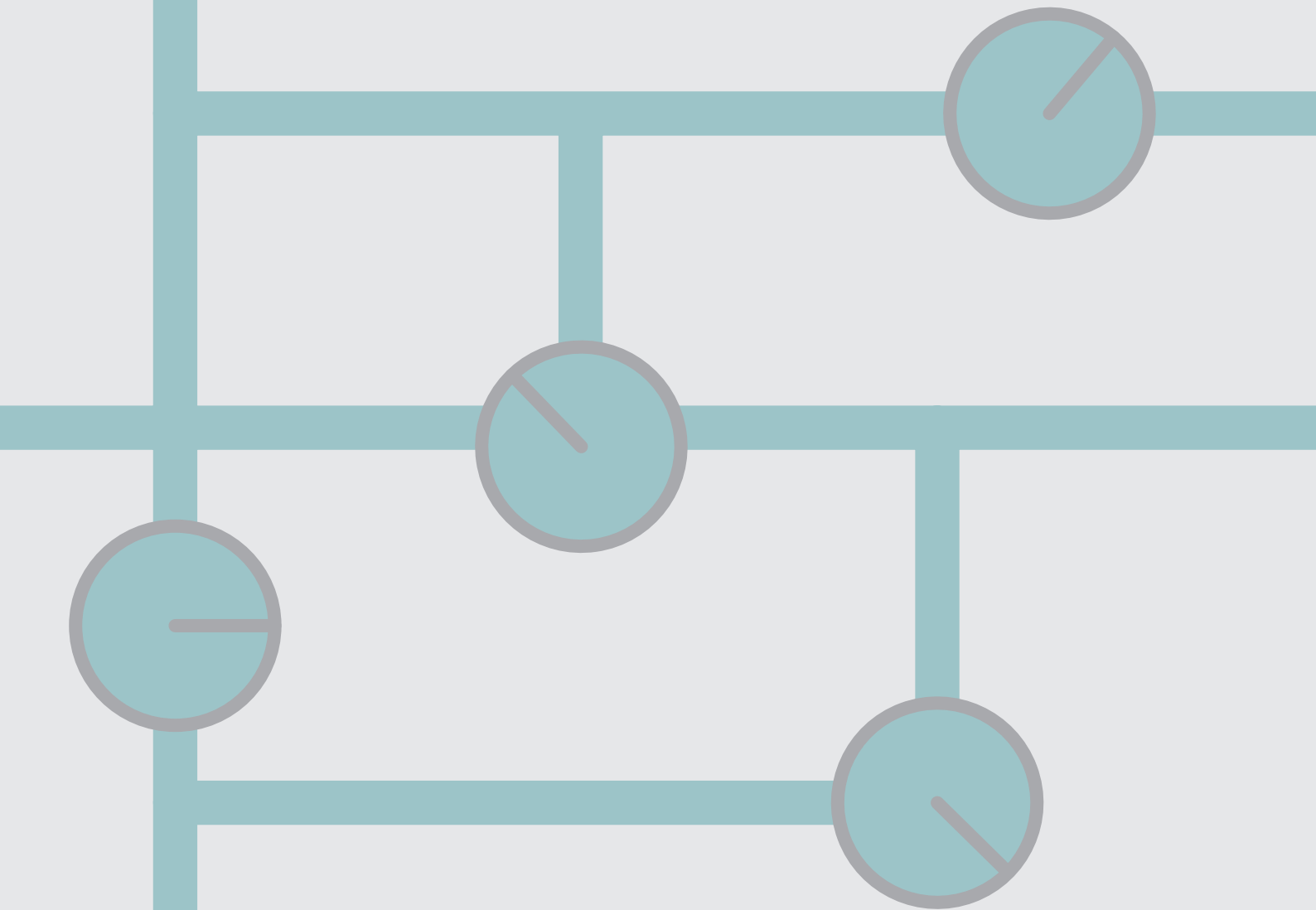
TESIS DOCTORAL

Análisis de psicoactivos en aguas, sedimentos y alimentos: de la epidemiología de alcantarilla a la forensía medioambiental



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Tesi Doctoral Internacional

Programa de doctorat 3108 en Contaminació,
Toxicologia i Sanitat Ambientals

Anàlisi de psicoactius en aigües, sediments i aliments: de la epidemiologia de claveguera a la forensía mediambiental

Análisis de psicoactivos en aguas, sedimentos y alimentos: de la epidemiología de alcantarilla a la forensia medioambiental

Analysis of psychoactive substances in water, sediments and food: from wastewater-based epidemiology to environmental forensic

Memòria presentada per optar al títol de Doctora per
María Jesús Andrés Costa

Dirigida per:

Dra. Yolanda Picó García

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Burjassot, febrer 2017

Vicente Andreu Pérez, Científico Titular del Consejo Superior de Investigaciones Científicas (CSIC) y Yolanda Picó García, Catedrática del área de Nutrición y Bromatología en el Departamento de Medicina Preventiva de la Universitat de València, ambos Doctores en Farmacia e investigadores del Centro de Investigaciones sobre Desertificación (CIDE):

I N F O R M A N :

Que la Licenciada *María Jesús Andrés Costa* ha realizado bajo nuestra dirección la Tesis Doctoral con mención internacional que lleva por título "**ANÁLISIS DE PSICOACTIVOS EN AGUAS, SEDIMENTOS Y ALIMENTOS: DE LA EPIDEMIOLOGÍA DE ALCANTARILLA A LA FORENSÍA MEDIOAMBIENTAL**" que se presenta como un compendio de diez publicaciones indexadas en el JCR o Scopus:

- Andrés-Costa, M. J., Rubio-López, N., Morales Suárez-Varela, M., and Pico, Y. (2014). **Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)**. *Environmental Pollution* **194**, 152-162. [**JCR (WOS)** IF 4.775 (2014) en el área de Ciencias Ambientales 17/223 Q1 (primer decil)]
- Álvarez-Ruiz, R., Andrés-Costa, M. J., Andreu, V., and Picó, Y. (2015). **Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter**. *Journal of Chromatography A* **1405**, 103-115. [**JCR (WOS)** IF 3.926 (2015) en el área de Química Analítica 14/77 Q1]
- Andrés-Costa, M. J., Andreu, V., and Picó, Y. (2016). **Analysis of psychoactive substances in water by information dependent acquisition on a hybrid qudrupole time-of-flight mass spectrometer**. *Journal of Chromatography A* **1461**, 98-106. [**JCR (WOS)** IF 3.926 (2015) en el área de Química Analítica 14/77 Q1].
- Andrés-Costa, M. J., Carmona, E., and Picó, Y. (2016). **Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight**. *Methods X* **3**, 307-314.[**SJR (Scopus)** 0.249 (2015)].
- Andrés-Costa, M. J., Escrivá, Ú., Andreu, V., and Picó, Y. (2016). **Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker.** *Science of The Total Environment* **541**, 616-622. [**JCR (WOS)** IF 3.976 (2015) en el área de Ciencias Medioambientales 32/225 Q1]
- Rico, M., Andrés-Costa, M. J., and Picó, Y. (2017). **Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study**. *Journal of Hazardous Materials* **323**, Part A, 156-165. [**JCR (WOS)** IF 4.836 (2015) en el área de Ciencias Medioambientales 19/225 Q1 (primer decil)]

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- Escrivá, Ú., Andrés-Costa, M.J., Andreu, V., Pico, Y. **Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety.** *Food Chemistry* **228**, 177-185 [**JCR (WOS)** IF 4.052 (2015) en el área de Ciencia y Tecnología de los Alimentos 7/121 Q1 (primer decil)]
- Andrés-Costa, M.J., Andreu, V., Pico, Y. (2017) **Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology,** TrAC-Trends Anal. Chem. (Enviada) [**JCR (WOS)** IF 7.487 (2015) en el área de Química Analítica 2/77 Q1 (primer decil)]
- Andrés-Costa, M.J., Proctor, K., Sabatini, M., Gee, A.P., Lewis, S.E., Pico, Y, Kasprzyk-Hordern, B. **Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance**, *Water Research* (Enviada) [**JCR (WOS)** IF 5.991 (2015) en el área de Ciencias Medioambientales 7/225 Q1(primer decil)]
- Andrés-Costa, M.J., Pascual-Aguilar, J.A., Andreu, V., Picó, Y. A**ssessing drugs of abuse distribuiton in Turia River based on geographic information system and liquid chromatography mass spectrometry**, *Environmental pollution* (En fase de revisión) [**JCR (WOS)** IF 3.976 (2015) en el área de Ciencias Medioambientales 32/225 Q1].

De estos trabajos, 7 han sido ya publicados y 3 están todavía en alguna fase del proceso editorial. La doctoranda es la primera autora de 7 de ellos (incluyendo 4 de los ya publicados) y la segunda de 3 de los trabajos presentados [dado que alguna parte de los mismos formó parte de tres trabajos de fin de master (TFM) cuyos autores firman en primer lugar]. En todos ellos, María Jesús ha realizado la mayor parte o todo el trabajo experimental, vigilando y supervisando estrechamente los experimentos cuando estos han formado parte de un TFM así como ha procedido al análisis de los resultados y la elaboración de los manuscritos en directa colaboración con nosotros. Sólo uno de los trabajos incluidos Andrés-Costa, M. J., Carmona, E., and Picó, Y. (2016). *Methods X* **3**, 307-314, formará también parte de la tesis de E. Carmona, dando que ambos han trabajado a partes iguales, siendo una interesante colaboración entre dos líneas de investigación que se desarrollan dentro del grupo, y dado que, sin contar con este trabajo, se cumplen los requisitos para la defensa de una tesis como compendio de publicaciones.

Moncada, 31 de enero de 2017

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ÍNDICE

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PRESENTACIÓN MEMORIA

OBJECTIUS I ESTRUCTURA

Les drogues d'abús tant lícites com il·lícites, els fàrmacs i els productes d'higiene personal són contaminants emergents, l'impacte dels quals és encara escassament conegut per als ecosistemes. En les últimes dècades hi ha hagut un creixent interès en l'estudi d'aquestes substàncies a causa del seu elevat consum i contínua descàrrega en compartiments mediambientals, després de la seua insuficient eliminació en les estacions depuradores d'aigües residuals (WWTP). La naturalesa i quantitat d'aquests contaminants estan freqüentment relacionats amb les característiques, estat de salut i hàbits de les poblacions que aboquen a aquestes WWTPs. Per tot açò, l'estudi d'aquests contaminants té una doble vessant: epidemiològicament es poden avaluar la magnitud, la naturalesa i els patrons de consum a través del ventall de compostos detectats en les WWTPs, i ambientalment es pot seguir el rastre i gestionar el perill que aquestes substàncies representen per als ecosistemes.

L'epidemiologia basada en l'anàlisi d'aigües residuals o també anomenada *epidemiologia de claveguera* constitueix una eina important per a l'estimació del consum local a través de la recerca dels fluxos de massa de drogues d'abús inalterades o dels seus metabòlits. L'EMCDDA *(European Monitoring Centre for Drugs and Drug Addiction*) reconeix aquest mètode com una alternativa als mètodes oficials basats en entrevistes, dades mèdiques i estadístiques criminals per a establir el consum de les drogues d'abús.

Els efectes nocius que el consum de drogues té per a la salut humana permeten predir que la presència d'aquests compostos en les aigües superficials pot tenir conseqüències toxicològiques sobre la flora i la biota aquàtiques. No obstant açò, una de les eines que encara no s'ha desenvolupat suficientment per abordar tots aquests estudis, són les tècniques de determinació d'aquests compostos en diferents matrius ambientals. A la complexitat de la matriu, se li uneixen les baixes concentracions i la inestabilitat d'aquests compostos, que fa difícil la seua determinació.

Per tot açò, l'**Objectiu General** s'ha centrat en desenvolupar mètodes analítics més fiables per (i) determinar la presència de les drogues estudiades en diferents matrius ambientals, (ii) per establir els patrons de consum d'aquestes substàncies a València i la seua àrea metropolitana, mitjançant *l'epidemiologia de claveguera* i, (ii) per a conèixer les fonts, nivells, transport i destinació de les mateixes en el medi ambient aplicant estratègies de *forensia mediambiental*, tenint en ment com a finalitat última com podrien afectar a l'ésser humà.

Els **Objectius Específics** de la present Tesi Doctoral s'han concretat en els següents punts:

1. Desenvolupar metodologies analítiques selectives i sensibles d'identificació i quantificació basades en la cromatografia líquida acoblada a l'espectrometria de masses (LC-MS/MS) i en la ultra alta resolució acoblada a l'espectrometria de masses amb un sistema híbrid de tipus quadrupol temps de vol (UHPLC-QqTOF-MS/MS) per determinar les substàncies seleccionades.

2. Aplicar les metodologies desenvolupades en l'anàlisi d'aigües (influents i efluents de les WWTPs i aigües superficials), sòlids en suspensió presents en els influents de les aigües residuals, fangs deshidratats elaborats en els tractaments de les WWTPs i sediments dipositats per les aigües superficials.

3. Establir els biomarcadors humans capaços d'estimar la grandària poblacional als quals proveeix cada WWTP, que oferisquen dades fiables i comparar-los amb els mètodes tradicionals usats fins al moment amb aquest efecte.

4. Determinar el consum de drogues lícites i il·lícites a nivell poblacional mitjançant l'anàlisi d'aigües residuals a partir de l'excreció de les drogues inalterades o els seus metabòlits.

5. Analitzar l'eficàcia de les WWTPs en el tractament de les aigües residuals, segons el grau d'eliminació de determinades drogues en els efluents.

6. Comprovar la influència i els efectes de la pressió humana en una conca hidrogràfica típica de l'àrea mediterrània a través d'un estudi integrat de la contaminació de les aigües per drogues i la seua relació amb altres característiques de qualitat de les aigües.

7. Estimar la bio i fotodegradació i transformació dels estereoisòmers d'una substància psicoactiva (fluoxetina) en diferents matrius (aigües superficials i fangs actius d'estacions depuradores).

8. Avaluar la toxicitat de determinades drogues a diferents nivells tròfics mitjançant estudis *in silico* i *in vivo*.

9. Estimar altres possibles rutes d'exposició humana a través dels aliments i els seus efectes potencials en la població.

Per desenvolupar aquests objectius, s'ha dissenyat el següent **Pla de Treball**:

En primer lloc, per definir amb claredat els objectius i establir els aspectes limitants d'aquesta temàtica s'ha realitzat una revisió profunda de la literatura publicada fins al moment, que ens ha permès conèixer els punts forts i febles per desenvolupar un treball amb perspectives de futur.

Les drogues d'abús, tant lícites com il·lícites, es van seleccionar atenent a un consum considerat com a tradicional o per estar catalogades com a noves substàncies psicoactives (NPS) —que inclouen tant les il·legalitzades com les que encara no estan subjectes a fiscalització internacional (conegudes com a *legal highs*)— comercialitzades a través d'Internet. Així mateix, es van seleccionar certs fàrmacs i productes d'higiene personal pel seu elevat interès com a contaminants emergents.

Seguidament es van dissenyar les campanyes de mostreig en les WWTPs de Pinedo I, Pinedo II i Quart-Benàger (influents, efluents i fangs deshidratats) i en la conca del riu Túria (aigües superficials i sediments). Durant el treball realitzat en la Universitat de Bath (Anglaterra) es van dissenyar les campanyes de mostreig dels fangs actius de la WWTP i les aigües superficials del riu Avon. A més, es van seleccionar diferents aliments que potencialment podrien contenir alguna de les substàncies objecte d'estudi.

Posteriorment es van desenvolupar i validar mètodes analítics per determinar les drogues tant lícites com il·lícites, els fàrmacs i productes d'higiene personal seleccionats. Aquests mètodes analítics es basen en les següents tècniques:

- Extracció en fase sòlida (SPE), extracció sòlid-líquid (SLE) i extracció assistida per ultrasons (UAE).

- LC-MS/MS utilitzant tant un analitzador triple quadrupol com un quadrupol temps de vol.

Finalment, els mètodes desenvolupats es van aplicar a les mostres de les matrius seleccionades, en les quals aquests compostos són susceptibles de ser transformats, mitjançant processos de degradació, en un ampli ventall de substàncies desconegudes, incrementant la complexitat de la seua determinació. Per tancar el cicle de la contaminació mediambiental i de com poden afectar aquestes drogues a l'ésser humà, hem de considerar que moltes d'elles provenen de plantes naturals, i que a més del seu consum intencionat poden arribar a l'ésser humà per altres vies.

La present Tesi Doctoral s'ha estructurat en 5 capítols. En cadascun dels capítols es presenten les publicacions científiques dutes a terme, les quals presenten la mateixa estructura interna: introducció, materials i mètodes, resultats i discussió, conclusions, referències i material complementari.

El **Primer Capítol** presenta una introducció general, a través d'una revisió bibliogràfica, sobre el paper que té l'espectrometria de masses com a eina en l'anomenada *epidemiologia de claveguera*. Aquesta revisió bibliogràfica cobreix tant l'estimació del consum de drogues d'abús com l'anàlisi de les estimacions poblacionals mitjançant biomarcadors, passant per l'estudi dels mètodes d'anàlisis no dirigides per establir el patró d'aquestes substàncies i els seus productes de transformació. A més, s'estableixen les perspectives futures de l'espectrometria de masses en aquesta matèria. Aquesta introducció s'estructura com un article de revisió:

- Publicació científica 1. *Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology (enviada)*

En el **Capítol 2** es detallen les metodologies analítiques desenvolupades i validades per determinar les substàncies seleccionades en diferents matrius ambientals (aigües, fangs actius i sòlids en suspensió procedents de WWTPs, i aigües superficials i sediments procedents de la conca del riu Túria). Les metodologies desenvolupades basades en l'anàlisi LC-MS/MS i UHPLC-QqTOF-MS/MS s'utilitzaren per analitzar els compostos seleccionats. Els resultats obtinguts dins d'aquest capítol s'estructuren en tres publicacions focalitzades en el desenvolupament de mètodes d'identificació i anàlisi d'aquests compostos.

- Publicació científica 2. *Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter*
- Publicació científica 3. *Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer*
- Publicació científica 4. *Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight*

En el **Capítol 3** s'apliquen les metodologies desenvolupades en el capítol anterior a l'anàlisi de les aigües procedents dels influents i efluents de

les WWTPs, per determinar la incidència de les drogues d'abús, estimar el seu consum en l'àrea metropolitana de València i establir el rendiment d'eliminació d'aquests compostos en les principals WWTPs involucrades. A més, s'avalua la toxicitat de determinades drogues a diferents nivells tròfics mitjançant estudis teòrics (ECOSAR). A partir de les dades obtingudes dels influents s'estableix el consum de drogues per part de la població proveïda per aquestes WWTPs, aplicant l'*epidemiologia de claveguera*. Així mateix es proposa un mètode alternatiu basat en l'anàlisi de biomarcadors presents en les aigües residuals per estimar aquesta població. Aquest capítol s'ha estructurat en tres publicacions científiques, les dues primeres focalitzades en l'estimació de drogues i alcohol mentre que la tercera se centra en valorar els mètodes per estimar la població servida per la WWTP, i on s'analitzen, a més de pels mètodes clàssics, diferents biomarcadors recentment proposats com a alternatives adequades per a aquesta estimació.

- Publicació científica 5. *Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)*
- Publicació científica 6. *Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker*
- Publicació científica 7. *Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study*

En el **Capítol 4** s'aborden els problemes mediambientals suscitats per aquests compostos, mitjançant un estudi integrat de la contaminació generada. En aquest apartat, s'analitzen nombrosos aspectes produïts a causa d'aquesta contaminació. La relació entre la presència d'aquests compostos i les característiques de la població s'estableix mitjançant l'ús d'un Sistema d'Informació Geogràfica (SIG). La relació entre els nivells d'aquests compostos i els paràmetres de qualitat d'aquestes aigües superficials es determina mitjançant les correlacions de Pearson. En aquest capítol, es presenta el treball dut a terme en la Universitat de Bath, a Anglaterra, durant

un període en el qual es va estimar la bio i fotodegradació i la transformació enantioselectiva d'un antidepressiu com és la fluoxetina en aigües superficials del riu Avon i fangs actius de la WWTP corresponent a aquella àrea. En aquest estudi també es va avaluar la toxicitat de la fluoxetina a diferents nivells tròfics mitjançant estudis *in vivo*. En la part final del capítol, s'avalua el possible impacte en l'ésser humà a causa del consum de diferents productes alimentaris que pogueren contenir cànnabis o els seus metabòlits. Aquest últim problema, derivat de l'alimentació del bestiar amb derivats del cànem, és un tema candent que ha preocupat a la *European Food Safety Authority* (EFSA), fins al punt de destacar en un comunicat, la necessitat d'aquests estudis (EFSA 2011). El capítol s'organitza en tres publicacions que cobreixen aquesta temàtica.

- Publicació científica 8. *Assessing drugs of abuse distribution in Turia River based on geographic information system and liquid chromatography mass spectrometry (enviada)*
- Publicació científica 9. *Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance (enviada)*
- Publicació científica 10. *Detection of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety*

El **Capítol 5** té com a objectiu aportar una visió global dels resultats i la discussió de la present Tesi Doctoral.

Finalment, s'arrepleguen les conclusions generals obtingudes i, a continuació, s'inclouen diversos **annexos** en els quals es troba la informació complementària (Índex de Taules, Índex de Figures i Índex d'Abreviatures).

Referències

EFSA (2011). "European Food and Safety Authority. Scientific Opinion on the safety of hemp (Cannabis genus) for use as animal feed." EFSA J **9**(3): 0-41. Available on-line: www.efsa.europa.eu/efsajournal.

Las drogas de abuso tanto lícitas como ilícitas, los fármacos y los productos de higiene personal son contaminantes emergentes cuyo impacto para los ecosistemas es todavía escasamente conocido. En las últimas décadas ha habido un creciente interés en el estudio de estas sustancias debido a su elevado consumo y continua descarga en compartimentos medioambientales, tras su insuficiente eliminación en las estaciones depuradoras de aguas residuales (WWTPs). La naturaleza y cantidad de estos contaminantes están frecuentemente relacionados con las características, estado de salud y hábitos de las poblaciones que vierten a estas WWTPs. Por todo ello, el estudio de estos contaminantes tiene una doble vertiente: epidemiológicamente se pueden evaluar la magnitud, naturaleza y patrones de consumo a través del abanico de compuestos detectados en las WWTPs y ambientalmente se puede seguir el rastro y gestionar el peligro que estas sustancias representan para los ecosistemas.

La epidemiología basada en el análisis de aguas residuales o también llamada *epidemiología de alcantarilla* constituye una herramienta importante para la estimación del consumo local a través de la investigación de los flujos de masa de drogas de abuso inalteradas o de sus metabolitos. La EMCDDA (*European Monitoring Centre for Drugs and Drug Addiction*) reconoce este método como una alternativa a los métodos oficiales basados en entrevistas, datos médicos y estadísticas criminales para establecer el consumo de las drogas de abuso.

Los efectos nocivos que el consumo de drogas tiene para la salud humana permiten predecir que la presencia de dichos compuestos en las aguas superficiales puede tener consecuencias toxicológicas sobre la flora y la biota acuáticas. Sin embargo, una de las herramientas, que todavía no se ha desarrollado suficientemente para abordar todos estos estudios, son las técnicas de determinación de estos compuestos en distintas matrices ambientales. A la complejidad de la matriz, se le unen las bajas

concentraciones y la inestabilidad de estos compuestos, que hace difícil su determinación.

Por todo ello, el **Objetivo General** propuesto se basa en el desarrollo de métodos analíticos más fiables para (i) determinar la presencia de las drogas estudiadas en diferentes matrices ambientales, (ii) para determinar los patrones de consumo de estas sustancias en Valencia y su área metropolitana, mediante la *epidemiología de alcantarilla* y (iii) para conocer las fuentes, niveles, transporte y destino de las mismas en el medioambiente aplicando estrategias de *forensía medioambiental*, teniendo en mente como fin último como podrían afectar al ser humano.

Los **Objetivos Específicos** de la presente Tesis Doctoral se han concretado en los siguientes puntos:

1. Desarrollar metodologías analíticas selectivas y sensibles de identificación y cuantificación basadas en la cromatografía líquida acoplada a la espectrometría de masas en tándem (LC-MS/MS), y en la cromatografía líquida de ultra alta resolución acoplada a la espectrometría de masas con un sistema híbrido de tipo cuadrupolo tiempo de vuelo (UHPLC-QqTOF-MS/MS) para determinar las sustancias seleccionadas.

2. Aplicar las metodologías desarrolladas en el análisis de aguas (influentes y efluentes de las WWTPs y aguas superficiales), sólidos en suspensión presentes en los influentes de las aguas residuales, lodos deshidratados elaborados en los tratamientos de las estaciones depuradoras y sedimentos depositados por las aguas superficiales.

3. Establecer los biomarcadores humanos capaces de estimar el tamaño poblacional a los que abastece cada WWTP que ofrezcan datos fiables y compararlos con los métodos tradicionales usados hasta el momento con este fin.

4. Determinar el consumo de drogas lícitas e ilícitas a nivel poblacional mediante el análisis de aguas residuales, a partir de la excreción de las drogas inalteradas o sus metabolitos.

5. Analizar la eficacia de las WWTPs en el tratamiento de las aguas residuales, según el grado de eliminación de determinadas drogas en los efluentes.

6. Comprobar la influencia y los efectos de la presión humana en una cuenca hidrográfica típica del área mediterránea a través de un estudio integrado de la contaminación de las aguas por drogas y su relación con otras características de calidad de las aguas.

7. Estimar la bio y fotodegradación y transformación de los estereoisómeros de una sustancia psicoactiva (fluoxetina) en diferentes matrices (aguas superficiales y lodos activos de estaciones depuradoras).

8. Evaluar la toxicidad de determinadas drogas a diferentes niveles tróficos mediante estudios *in silico* e *in vivo*.

9. Estimar otras posibles rutas de exposición humana a través de los alimentos y sus efectos potenciales en la población.

Para desarrollar estos objetivos, se ha diseñado el siguiente **Plan de Trabajo**:

En primer lugar, para definir con claridad los objetivos y establecer los aspectos limitantes de esta temática se ha realizado una revisión profunda de la literatura publicada hasta el momento, que nos ha permitido conocer los puntos fuertes y débiles para desarrollar un trabajo con perspectivas de futuro.

Las drogas de abuso tanto lícitas como ilícitas se seleccionaron atendiendo a un consumo considerado como tradicional o por estar catalogadas como nuevas sustancia psicoactivas (NPS) —que incluyen tanto las ilegalizadas como las que aún no están sujetas a fiscalización internacional (conocidas como *legal highs*)— comercializadas a través de Internet. Asimismo,

se seleccionaron ciertos fármacos y productos de higiene personal por su elevado interés como contaminantes emergentes.

Seguidamente se diseñaron las campañas de muestreo en las WWTPs de Pinedo I, Pinedo II y Quart-Benáger (influentes, efluentes y lodos deshidratados) y en la cuenca del río Turia (aguas superficiales y sedimentos). Durante el trabajo realizado en la Universidad de Bath (Inglaterra) se diseñaron las campañas de muestreo de los lodos activos de la WWTP y las aguas superficiales del río Avon. Además, se seleccionaron diferentes alimentos que potencialmente podrían contener alguna de las sustancias objeto de estudio.

Posteriormente se desarrollaron y validaron métodos analíticos para determinar las drogas tanto lícitas como ilícitas, los fármacos y productos de higiene personal seleccionados. Dichos métodos analíticos se basan en las siguientes técnicas:

- Extracción en fase sólida (SPE), extracción sólido-líquido (SLE) y extracción asistida por ultrasonidos (UAE).

- LC-MS/MS utilizando tanto un analizador triple cuadrupolo como un cuadrupolo tiempo de vuelo.

Por último, los métodos desarrollados se aplicaron a las muestras de las matrices seleccionadas, en las que estos compuestos son susceptibles de ser transformados, mediante procesos de degradación, en un amplio abanico de sustancias desconocidas, incrementando la complejidad de su determinación. Para cerrar el ciclo de la contaminación medioambiental y de cómo pueden afectar estas drogas al ser humano, debemos considerar que muchas de ellas provienen de plantas naturales, y que además de su consumo intencionado pueden llegar al ser humano por otras vías.

La presente Tesis Doctoral se ha estructurado en 5 capítulos. En cada uno de los capítulos se presentan las publicaciones científicas llevadas a cabo, las cuales presentan la misma estructura interna: introducción, materiales y métodos, resultados y discusión, conclusiones, referencias y material complementario.

El **Primer Capítulo** presenta una introducción general, a través de una revisión bibliográfica, sobre el papel que tiene la espectrometría de masas como herramienta en la llamada *epidemiología de alcantarilla*. Esta revisión bibliográfica cubre tanto la estimación del consumo de drogas de abuso, como el análisis de las estimaciones poblacionales mediante biomarcadores, pasando por el estudio de los métodos de análisis no dirigidos para establecer el patrón de estas sustancias y sus productos de transformación. Además se establecen las perspectivas futuras de la espectrometría de masas en esta materia. Esta introducción se estructura como un artículo de revisión:

- Publicación científica 1. *Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology (enviada)*

En el **Capítulo 2** se detallan las metodologías analíticas desarrolladas y validadas para determinar las sustancias seleccionadas en diferentes matrices ambientales (aguas, lodos activos y sólidos en suspensión procedentes de WWTPs, y aguas superficiales y sedimentos procedentes de la cuenca del río Turia). Las metodologías desarrolladas basadas en el análisis LC-MS/MS y UHPLC-QqTOF-MS/MS se utilizaron para analizar los compuestos seleccionados. Los resultados obtenidos dentro de este capítulo se estructuran en tres publicaciones focalizadas en el desarrollo de métodos de identificación y análisis de estos compuestos.

- Publicación científica 2. *Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter*

- Publicación científica 3. *Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer*
- Publicación científica 4. *Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight*

En el **Capítulo 3** se aplican las metodologías desarrolladas en el capítulo anterior al análisis de las aguas procedentes de los influentes y efluentes de las WWTPs, para determinar la incidencia de las drogas de abuso, estimar su consumo en el área metropolitana de Valencia y establecer el rendimiento de eliminación de dichos compuestos en las principales WWTPs involucradas. Además se evalúa la toxicidad de determinadas drogas a diferentes niveles tróficos mediante estudios teóricos (ECOSAR). A partir de los datos obtenidos de los influentes se establece el consumo de drogas por parte de la población abastecida por dichas WWTPs, aplicando la *epidemiología de alcantarilla*. Asimismo se propone un método alternativo basado en el análisis de biomarcadores presentes en las aguas residuales para estimar dicha población. Este capítulo se ha estructurado en tres publicaciones científicas, las dos primeras focalizadas en la estimación de drogas y alcohol mientras que la tercera se centra en valorar los métodos para estimar la población servida por la WWTP, y donde se analizan, además de por los métodos clásicos, distintos biomarcadores recientemente propuestos como alternativas adecuadas para dicha estimación.

- Publicación científica 5. *Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)*
- Publicación científica 6. *Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker*
- Publicación científica 7. *Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study.*

En el **Capítulo 4** se abordan los problemas medioambientales suscitados por estos compuestos, mediante un estudio integrado de la contaminación generada. En este apartado se analizan numerosos aspectos producidos a causa de esta contaminación. La relación entre la presencia de estos compuestos y las características de la población se establece mediante el uso de un Sistema de Información Geográfica (SIG). La relación entre los niveles de estos compuestos y los parámetros de calidad de estas aguas superficiales se determina mediante las correlaciones de Pearson. En este capítulo se presenta el trabajo llevado a cabo en la Universidad de Bath, en Inglaterra, durante un periodo en el cual se estimó la bio y fotodegradación y la transformación enantioselectiva de un antidepresivo como es la fluoxetina en aguas superficiales del río Avon y lodos activos de la WWTP correspondiente a esa área. En este estudio también se evaluó la toxicidad de la fluoxetina a diferentes niveles tróficos mediante estudios *in vivo*. En la parte final del capítulo se evalúa el posible impacto en el ser humano debido al consumo de distintos productos alimenticios que pudieran contener cannabis o sus metabolitos. Este último problema, derivado de la alimentación del ganado con derivados del cáñamo, es un tema candente que ha preocupado a la *European Food Safety Authority* (EFSA), hasta el punto de destacar en un comunicado, la necesidad de estos estudios (EFSA 2011). El capítulo se organiza en tres publicaciones que cubren esta temática.

- Publicación científica 8. *Assessing drugs of abuse distribution in Turia River based on geographic information system and liquid chromatography mass spectrometry (enviada)*
- Publicación científica 9. *Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance (enviada)*
- Publicación científica 10. *Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety*

El **Capítulo 5** tiene como objetivo aportar una visión global de los resultados y la discusión de la presente Tesis Doctoral.

Por último, se recogen las **conclusiones generales** obtenidas y, a continuación, se incluyen diversos **anexos** en los que se encuentra la información complementaria (Índice de Tablas, Índice de Figuras, Índice de Abreviaturas).

Referencias

EFSA (2011). "European Food and Safety Authority. Scientific Opinion on the safety of hemp (Cannabis genus) for use as animal feed." EFSA J **9**(3): 0-41. Available on-line: www.efsa.europa.eu/efsajournal.

AIM AND STRUCTURE

Licit and illicit drugs of abuse, pharmaceuticals and personal care products are emerging contaminants whose impact on ecosystems is still poorly understood. In the last decades there has been a growing interest in the study of these substances due to their high consumption and continuous discharges into environmental compartments, after their insufficient disposal in wastewater treatment plants (WWTPs). The origin and quantity of these pollutants are often related to the characteristics, health conditions and habits of the populations that pour into these WWTPs. Therefore, the study of these pollutants has a double aspect: the magnitude, origin and patterns of consumption can be evaluated epidemiologically through the range of compounds detected in the WWTP and environmentally by tracking and managing the hazard of these substances to ecosystems

Wastewater-based epidemiology analysis, also called *sewage epidemiology*, is an important tool for estimating local consumption through the study of mass flows of unchanged drugs of abuse or their metabolites. The EMCDDA (European Monitoring Centre for Drugs and Drug Addiction) recognizes this method as an alternative to official methods based on interviews, medical data and criminal statistics to establish the consumption of drugs of abuse.

The adverse effects of drug consumption on human health make it possible to predict that the presence of these compounds in surface waters could have toxicological consequences on aquatic flora and biota. However, one of the tools, that has not yet been sufficiently developed to address all these studies, are the techniques for determining these compounds in different environmental matrices. In addition to the complexity of the matrix, the low concentrations and the instability of these compounds make their determination difficult.

Therefore, **General Objective** has been to develop more reliable analytical methods to (i) determine the presence of studied drugs in different

environmental matrices, (ii) establish patterns of consumption of these substances in Valencia and its metropolitan area, through *sewage epidemiology* and (iii) know the sources, levels, transport and fate of these substances in the environment applying environmental forensics approaches, and keeping in mind how could they affect to humans as ultimate goal.

Specific Objectives of this Doctoral Thesis has been divided into the following aims:

1. Develop selective and sensitive analytical methodologies for identification and quantification based on liquid chromatography tandem mass spectrometry (LC-MS/MS) and ultra-high performance liquid chromatography hybrid quadrupole time-of-flight mass spectrometry (UHPLC-QqTOF-MS / MS) to determine selected substances.

2. Apply the developed methodologies in water analysis (influents and effluents of the WWTPs and surface waters), particulate material present in the influent of the wastewater, dehydrated sludge processed in the treatment of the treatment plants and sediments deposited by surface waters.

3. Establish human biomarkers capable of estimating the population size to which each WWTP supplies that offer reliable data and compare them with the traditional methods used so far for this purpose.

4. Determine the consumption of licit and illicit drugs at the population level by analyzing wastewater from the excretion of unchanged drugs or their metabolites.

5. Analyse the efficiency of treatment of wastewater in WWTPs, according to the percentage of elimination of drugs in effluents.

6. Verify the influence and effects of human pressure in a typical Mediterranean river basin through an integrated study of water contamination by drugs and its relation with other characteristics of water quality.

7. Estimate the bio- and photodegradation and transformation of the stereoisomers of a psychoactive substance (fluoxetine) in different matrices (surface water and active sludge from WWTP).

8. Evaluate the toxicity of certain drugs at different trophic levels by *in silico* and *in vivo* studies.

9. Estimate other possible routes of human exposure through food and their potential effects on population.

In order to develop these objectives, the following **Work Plan** has been designed:

Firstly, in order to define the aims clearly and to establish the limiting aspects of this topic, a thorough review of the literature published up to now has been carried out, which has allowed us to recognize the strengths and weaknesses to develop a future-oriented work.

Drugs of abuse were selected attending to a consumption considered traditional or by being in the group of new psychoactive substances (NPS) — including both, illegal and those not yet subject to international control (known as *legal highs*)— marketed via the internet. Certain pharmaceuticals and personal care products were selected because of their high interest as emerging pollutants.

Sampling campaigns were then designed in WWTPs of Pinedo I, Pinedo II and Quart-Benáger (influent, effluent and dehydrated active sludge) and in the Turia River basin (surface waters and sediments). During the work carried out at the University of Bath (England) the sampling campaigns of the activated sludge of the WWTP and the surface waters of the Avon River were designed. In addition, different foods were selected that could potentially contain some of the substances under study.

Afterwards, analytical methods were developed and validated to determine licit and illicit drugs, pharmaceuticals and personal care products. These analytical methods are based on the following techniques:

- Solid-phase extraction (SPE), solid-liquid extraction (SLE) and ultrasonic assisted extraction (UAE)

- LC-MS/MS using both a triple quadrupole and quadrupole time-of-flight analysers.

Finally, the developed methods were applied to the selected matrices where these compounds are susceptible to be transformed, through several degradation processes, in a wide range of unknown substances, increasing the complexity of their determination. To close the cycle of environmental pollution and how these drugs can affect humans, we must consider that many of them come from natural plants, and that, in addition to their intentional consumption, can reach humans by other ways.

This Doctoral Thesis has been structured into 5 chapters. Each chapter introduces the scientific publications carried out, which present the same internal structure: introduction, materials and methods, results and discussion, conclusions, references and complementary material.

The **First Chapter** presents a general introduction of the role that mass spectrometry can play as a tool in so-called *sewage epidemiology*. The review of the published literature covers the estimation of the use of licit and illicit drugs of abuse, the population estimation using biomarkers, and the study of wide-scope analytical methods to establish the pattern of these substances and their transformation products. In addition, the future prospects of mass spectrometry in this area are established. This introduction has been structured as a review article:

- Scientific publication 1. *Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology (sent)*

Chapter 2 details the development and validation of analytical methods to determine the selected substances in different environmental matrices (water, sludge and particulate matter from WWTPs, and surface waters and sediments from the Turia River basin). LC-MS/MS and UHPLC-QqTOF-MS/MS based methods were selected to analyse the selected compounds. The results obtained in this chapter are structured into three scientific publications focused on the development of methods to identify and quantify these compounds.

- Scientific publication 2. *Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter*
- Scientific publication 3. *Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer*
- Scientific publication 4. *Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight*

In **Chapter 3**, the methodologies developed in the previous chapter are applied to the analysis of WWTPs influents and effluents to determine the incidence of drugs of abuse, estimate their consumption in the metropolitan area of Valencia and establish the removal efficiency of selected WWTPs. Furthermore, the toxicity of certain drugs at different trophic levels is evaluated through theoretical studies (ECOSAR). The consumption of drugs of abuse by the population supplied by these WWTPs is established from the data obtained from the influents by applying *sewage epidemiology*. An alternative method based on the analysis of biomarkers in the wastewater is also proposed to estimate this population. This chapter has been structured in three scientific publications, the first two focused on the estimation of drugs and alcohol, while the third focuses on assessing the methods to estimate the

population served by the WWTP, both classical methods and recently proposed biomarkers are suitable alternatives to achieve this estimation.

- Scientific publication 5. *Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)*
- Scientific publication 6. *Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker*
- Scientific publication 7. *Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study.*

Chapter 4 addresses the environmental problems raised by these compounds through an integrated study of the generated pollution. In this section, a large number of aspects of this contamination are analyzed. The relationship between the presence of these compounds and the characteristics of the population is established through the use of a Geographic Information System (GIS). The relationship between the levels of these compounds and the quality parameters of these surface waters is determined by Pearson's correlations. This chapter presents the work carried out at the University of Bath, England, during a period in which the bio- and photodegradation and the enantioselective transformation of an antidepressant such as fluoxetine were estimated in the surface waters of the Avon River and activated sludge from the WWTP corresponding to that area. This study also evaluated the toxicity of fluoxetine at different trophic levels by *in vivo* studies. The final part of the chapter assesses the possible impact on humans due to the consumption of different food products that could contain cannabis or its metabolites. This last problem, derived from the feeding of livestock with hemp derivatives, is a hot topic that has worried the European Food Safety Authority (EFSA), up to the point of emphasizing in a statement the need for these studies. The chapter is organized into three scientific publications covering this subject (EFSA 2011). This chapter has been structured in three scientific publications.

- Scientific publication 8. *Assessing drugs of abuse distribution in Turia River based on geographic information system and liquid chromatography mass spectrometry (sent)*
- Scientific publication 9. *Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance (sent)*
- Scientific publication 10. *Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seeds to ensure food safety*

Chapter 5 aims to provide an overview of the results and discussion of this Doctoral Thesis.

Finally, the **general conclusions** are gathered and several annexes are included with **complementary information** (Index of Abbreviations, Index of Figures and Index of Tables).

References

EFSA (2011). "European Food and Safety Authority. Scientific Opinion on the safety of hemp (Cannabis genus) for use as animal feed." EFSA J **9**(3): 0-41. Available on-line: www.efsa.europa.eu/efsajournal.

Capítulo 1

CAPÍTULO 1

INTRODUCCIÓN

El Primer Capítulo presenta una introducción general, a través de una revisión bibliográfica, sobre el papel que tiene la espectrometría de masas como herramienta en la llamada *epidemiología de alcantarilla*. Esta revisión bibliográfica cubre tanto la estimación del consumo de drogas de abuso, como el análisis de las estimaciones poblacionales mediante biomarcadores, pasando por el estudio de los métodos de análisis no dirigidos para establecer el patrón de estas sustancias y sus productos de transformación. Además se establecen las perspectivas futuras de la espectrometría de masas en esta materia. Esta introducción se estructura como un artículo de revisión.

- Publicación científica 1. *Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology*

PUBLICACIÓN CIENTÍFICA 1

Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology

M.J. Andrés-Costa, V. Andreu, Y. Picó

TrAC-Trends Anal.Chem (Enviada)

Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology

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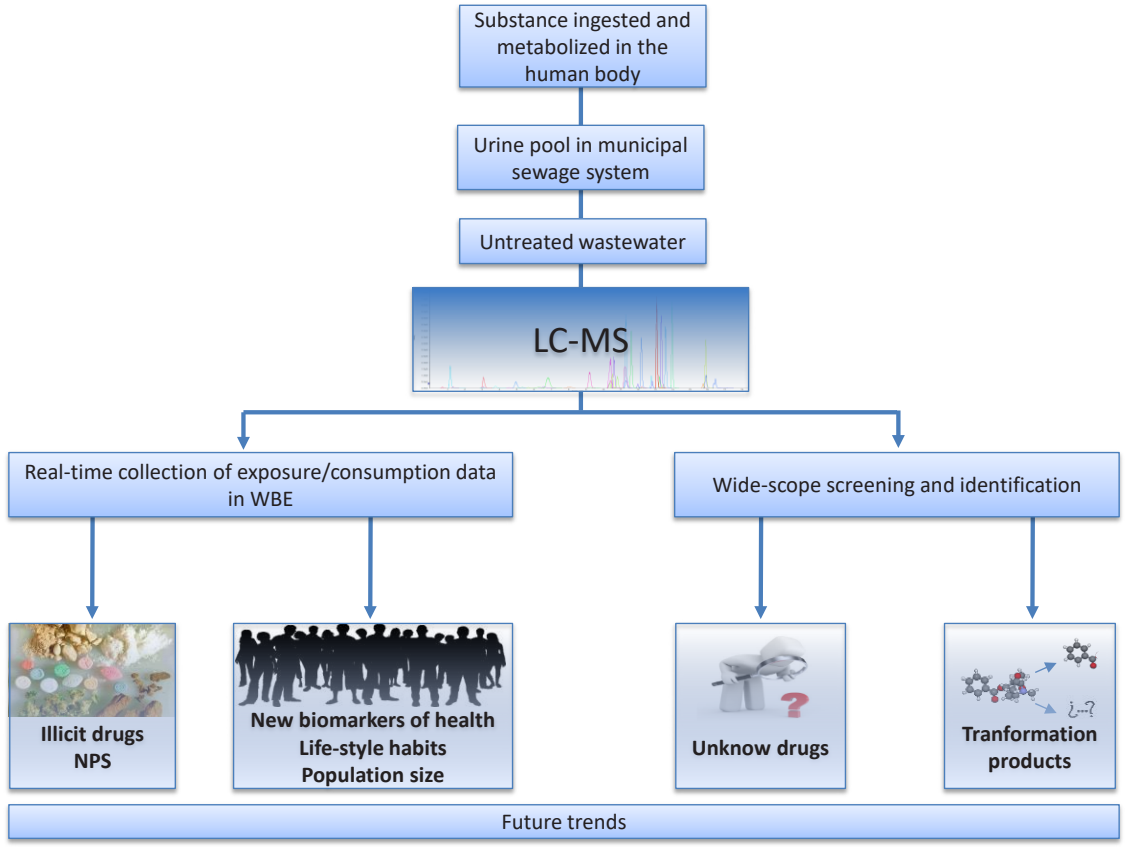
Abstract

Wastewater-based epidemiology (WBE) was devised by Daughton in 2001 and led to the practice by Zuccato et al. in 2005 to determine consumption of illicit drugs. Many researchers are focused on investigation of population habits through wastewater analysis due their ability to estimate substance consumption and unique capacity to assess biomarkers of different contaminants and health states. WBE has started to develop significantly in the last 3 years mainly due to advancement of liquid chromatography-mass spectrometry (LC-MS). LC coupled to time-of-flight and Orbitrap high-resolution-MS promise to extend knowledge on major metabolites and degradation products of targets as well as on health and disease biomarkers. This overview introduces recent methods and outstanding challenges in the application of LC-MS to WBE, and summarizes trends that involved LC-MS, including suspect and non-target screening and proposed workflows for discovering drugs metabolites and biomarkers. Further challenges for LC-MS analyses related to WBE are also discussed.

Keywords: Wastewater-based epidemiology; Liquid chromatography-mass spectrometry; Illicit drugs consumption; Population biomarkers; High-resolution mass spectrometry.

Graphical abstract



Contents

1. Introduction

Wastewater-based epidemiology (WBE) follows from the principle of considering untreated wastewater, which end-up in the municipal sewage system, as a huge urine and stools pool where the unaltered compound and/or metabolic residues of any substance ingested in the human body or excreted as a part of catabolic reactions are present [1-11]. WBE was conceived by Daughton in 2001 [12] and implemented using cocaine as a model compound by Zuccato et al. (2005) [13]. Cocaine and its main urinary metabolite (benzoylecgonine, BE) were measured by liquid chromatography mass spectrometry (LC-MS) in water and wastewater. Since then, WBE and LC-MS have been strongly linked so that, the evolution of the former has marched hand in hand with the instrumental development of the latter and the implantation of their new platforms and improved workflows [1, 6, 7]. This methodology —schematized in **Fig. 1.1**— has turned into an important tool for monitoring patterns and trends of illicit drug consumption in communities that allows to track human habits and lifestyle as well as the associated outcomes on health, education and crime [11]. Sewage analysis CORe group Europe (SCORE) was established in 2010 to collaborate on international studies comparing illicit drug use between major cities and evaluate the analytical procedures to their determination in wastewater [14]. WBE is also the target of several European actions including the COST Action ES1307 “Sewage biomarker analysis for community health assessment” and other similar or complementary initiatives [14]. The European Monitoring Centre for Drug and Drug Addiction (EMCDDA) and other international governmental agencies such as the United Nations Office on Drugs and Crime, have shown interests in exploring the potential of wastewater analysis for enhancing drug monitoring in Europe [15, 16]. Recently, and already for several years, EMCDDA supports a Europe-wide demonstration program “Wastewater analysis and drugs — a

European multi-city study" that includes year after years an increasing number of cities [15]. The results are released through an innovative interactive map and chart-based tool allowing the user to look at geographical and temporal patterns and zoom in on results per city. These are just few highlights to remark the interest of this approach fully based on LC-MS determination.

A number of reviews cover, totally or in part, the subject of the WBE. Most of them are focused on how to calculate drug consumption from wastewater analysis and the reliability of the estimation [8, 11, 17, 18]. Other large group of reviews evaluate the uncertainties associated with determination of community drug use through the measurement of sewage drug biomarkers [2, 3, 18, 19]. A number of original research articles that, which compared illicit drug use in increasing number European cities through sewage analysis, complemented previous reviews [9, 20]. This contrasts with the scarce reviews that cover the analytical part of these determinations. Only one recent review has covered the most-recent literature available (mostly from the last 5 years) on the mass spectrometric determination of biomarkers of illicit drug in wastewater with particular emphasis on the different analytical strategies applied [21], which is only a small part of WBE.

It should be noted that recent advances in LC-MS has allowed to extend WBE to estimate consumption, use or exposure to different licit and illicit drugs or even environmental stressors [5, 10]. In a near future, WBE is also envisaged as a promising tool for the real-time collection of exposure/effects data that reflects the overall average health of entire communities. These achievements are related to the advances on LC-high resolution (HR)-MS, including the recently developed schemes based on suspected screening and non-target searching that open a new horizon to detect new compounds and identify not-yet-reported metabolites and degradation products [22, 23].

The present review critically addresses the current state-of-the-art in recent advances in LC-MS and LC-HR-MS that are applied in WBE and their pros and cons. A selection of the most significant papers recently published on instrumental and methodological aspects, and the newest applications are included. There are a huge number of applications in this field ranging from developing LC-MS to estimate drug consumption to stability and uncertainty studies, so we examine the relevant studies published in the last 3–4 years. First, conventional LC-MS aspects in real time collection of exposure/consumption data are discussed as well as their applications to calculate population size. Next, an insight into the technologies that need further exploration and advancement in the near future for effective discovering and detection of a wider range of biomarkers in order to enlarge the scope of WBE is provided.

2. LC-MS for real-time collection of exposure/consumption data in WBE

2.1. Illicit Drugs and New Psychoactive Substances (NPS)

To the moment, WBE has been widely applied to evaluate the illicit drugs use patterns. Several reviews cover this topic. Van Nuijs et al. (2011) [11] detailed the illicit drug consumption estimations derived from wastewater analysis published until 2010. Castiglioni et al. (2008) [18] focused attention on current research gaps and requirements to bring together wastewater analysis and drug epidemiology. Hernandez et al. (2016) [21] emphasised on the different mass spectrometry strategies applied to identify and quantify illicit drug biomarkers in the last five years. Here, the large number of publications evaluating traditional illicit drug consumption in different cities of the five continents, for the last 5 years is compiled in the supplementary material (**Table S1.1**) to offer together with the mentioned reviews an overview to the reader. The analytical protocols are already well-established for those considered “traditional

illicit drugs". Nowadays, the pattern of these compounds changes continuously since uncontrolled new psychoactive substances (NPS) ("legal highs", "designer drugs", "bath salts", etc.), not regulated by current laws are proliferating in number and variety. These new drugs, even those that involve minor modification of the chemical structure of established ones, have resulted in a continual analytical challenge for their detection, identification and measurement undertaken in some regards through WBE. **Table 1.1** outlines NPS determined by the already existing analytical schemes for WBE (detailed information of the methods is in **Table S1.1** of SI).

The sample preparation protocol requires, at least, the isolation and concentration of the illicit drugs, mostly performed by solid phase extraction (SPE). The most employed sorbents for SPE are either hydrophilic-lipophilic balanced (HLB) reversed-phases or the mixed-mode (commonly with a cation exchanger) modification of them that achieve quantitative extraction of the illicit drugs and eliminate the influence of the matrix components. The most important difference between both extraction protocols is that the mixed mode required to acidify the sample to pH ca. 3 to ensure that analytes are positively charged. Cathinones, phenetylamines, piperazines and synthetic cannabinoids were solid-phase-extracted using mixed reverse-phase cation-exchange cartridges [25, 28-32, 34, 37] but the optimized conditions for the analysis of the whole set of compounds mainly used polymeric or hydrophilic/lipophilic sorbent [24, 26, 33]. This is somehow contradictory because several studies that compared the performance of Oasis HLB and the mixed mode cartridges have reported always better recoveries, reduction in matrix interferences, and improved peak shapes for the majority of the compounds using the mixed modes [30, 32, 35].

Both, conventional LC and ultra-high performance (UHP)LC methods are equally proposed for the analysis of illicit drugs in wastewaters, a variety of

chromatography separation columns has been used, usually reverse-phase ones with different stationary phases as C_{18} and phenyl as well as in HILIC phases. The NPS have mostly been determined using reversed phase C_{18} columns [24, 25, 28-34]. However, the phenethylamine-based compounds (BUTL, ETONE, METL, PMA, PMMA and MPA) are very polar and thus retained well on HILIC [35]. Column selection for chromatographic analysis is an important step since the structures and physicochemical properties of the different NPS families are very different among them. As an example, Borova et al. (2015) [26] compared three different reversed phase columns, consisted of silica particles and high strength silica particles with the same trifunctional alkyl C_{18} ligand, and a pentafluorophenyl (core shell silica and a pentafluorophenyl ligand) in terms of chromatographic peak shape and resolution for 1-Benzylpiperazine, JWH-073, JWH-018, MEP and MPPP. **Fig. 1.2** illustrates the obtained chromatograms. Stronger retention due to the π–π bonding was observed for the polar compounds when using a PFP column compared to the C_{18} columns. Chiral-CBH has been used to perform the separation of enantiomeric compounds. Although not tested yet for NPS, the capacity to undertake enantiomeric analysis of chiral illicit drugs is of vital importance in studies within both, the environmental and WBE fields. Different mobile phases were composed of water and an organic solvent (methanol or acetonitrile) with modifiers such as ammonium formate, ammonium acetate, trimethylamine and acids (formic or acetic) to improve the ionization of compounds that are monitored mostly in positive ionization mode or to ensure appropriate peak shape. The combination methanol-water or acetonitrile water with formic acid as mobile phase showed the best chromatographic performance, when working in positive ionization (PI) mode, in studies dealing with NPS [24-27, 29-36].

Illicit drugs and their metabolites have been determined by a variety of mass spectrometers using electrospray ionization (ESI), frequently in positive ionization mode with the exception of 11-nor-9-carboxy-Δ9-tetrahidrocannabinol (THC-COOH) that ionizes also in negative mode. Mass spectrometers include low resolution (LR) [triple quadrupole mass spectrometer (QqQ), quadrupole linear ion-trap (QTRAP)] and HR instruments [hybrid quadrupole Orbitrap and quadrupole time-of-flight (QqTOF)]. LC-MS/MS or UHPLC-MS/MS methods using QqQ or QTrap mass analyzers are often proposed for the determination of illicit drugs because of their high sensitivity in the selected reaction monitoring (SRM) acquisition mode (**Table S1.1**) that monitors specific precursor ion → product ion transitions. The largest part of the MS/MS instruments but, especially, the newest ones have the ability to scan simultaneously many *m/z* transitions at high frequency (up to 0.02 sec/scan) as well as the option to program several segments or to monitor different *m/z* transitions for a short time interval before and after the analyte's retention time. This is the most common scheme even for NPS [26-28, 30-32, 34-36]. The improved analytical performances provided by current LC-MS/MS instruments allow the detection of extremely low illicit drugs levels in wastewater, never achieved so far.

QqTOF and Orbitrap provided the full mass spectrum and achieve a resolution of >30,000 and mass accuracy below 5 ppm that distinguish between the target compound and other isobaric interferences, and provide a tentative identification of the compounds based on accurate mass assignments. Compared with QqTOF, Orbitrap has higher resolution at low m/z, but slower rate of data acquisition. These instruments have enlarge the working modes in LC-MS as illustrated in **Fig.1.3**. In this section, we will only outlined the target results (with standard analytes selected a priori) that are commonly quantified extracting ion chromatograms (XICs) of a narrow *m/z* range (5-20

mDa). Nowadays there are two different acquisition strategies—data dependent acquisition (DDA) and data independent acquisition (DIA). DDA involves a software-controlled switch from MS to MS/MS and back again being a real MS/MS. Full scan MS is used as survey scan to select the precursor ions of interest (of a specific m/z value, over an intensity threshold, etc.) for subsequent MS/MS experiments. Different strategies, such as 'dynamic exclusion' and 'background subtraction' prevent the reselection of precursor ions increasing the number of compounds fragmented. In DIA, several full scans using different conditions (e.g. collision energies) are collected simultaneously by either (1) fragmenting all ions entering the mass spectrometer (e.g. broadband DIA, MS^E or MS^{All}) or (2) dividing the full *m/z* range into fixed smaller *m/z* isolation windows that are independently and consecutively analyzed (e.g. 'Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra', SWATH). HR mass spectrometers have been successfully employed to the analysis of target NPS in wastewater [24, 25, 33]. For instance, Andres-Costa et al. (2016) [24] quantitatively determined of 42 illicit drugs, NPS and metabolites in wastewater using a QqTOF with the simultaneous acquisition of MS/MS by DDA using just a threshold intensity of 1000 cps together with dynamic background subtraction and an exclusion list of already known interferences. The lower calibration levels achieved were between 1 and 100 ng L^{-1}. At these levels, target compounds provide a chromatographic peak of appropriate peak-shape and the MS/MS was obtained. However, in another study, Gonzalez-Mariño et al. (2016) [25] designed a suspect screening and a target method approach and compared them for 35 synthetic cannabinoids and cathinones using a linear ion trap (QTRAP)-orbitrap. **Fig.1.4** illustrated the comparison the of DDA using a full scan MS (150–600m/z), and 5 events where MS^2 scans of the 5 most intense m/z recorded in the first event (DDA TOP 5) with a target method that fragment specific and predefined

precursor ions (five or six depending on the method) after the first full-scan MS event. Using DDA, the lowest concentration showed in some cases an unsuccessful match for fragment ions, isotopic pattern, and library search. This is due to the fact that the m/z of the ionized analytes were not among the five most intense recorded in the first event, so they were not submitted to fragmentation. The field of HR-MS is surely a growing one, although there are still few applications. It is, however, only a matter of time and resources before many practical results and a more rational use of the equipment capabilities will be reached.

The old and well-known disadvantage of LC-MS, in any of its different platforms, is the matrix effect that can result in ion suppression or ion enhancement. The setting-up of assays by MS has become greatly facilitated by the availability of stable isotopically-labelled Internal Standards (IS) (deuterium, ^{13}C, ^{15}N) that compensate for deleterious matrix effects variably affecting influent wastewater samples, which may otherwise compromise the accuracy of the analytical method. These IS are also important for the accurate quantification of analytes that are quantified simultaneously but cannot be chromatographically separated. Several strategies using IS and surrogates have been proposed. No single “gold standard method” can solve all of the analytical problems associated with illicit drug quantification in wastewater. The most appropriate method must be evaluated for any particular applications.

2.2. New biomarkers of health, life-style habits and population size

In a near future, WBE is also envisaged as a promising tool for the real-time collection of exposure/effects data that reflects the overall average health of entire communities using specific biomarkers [5, 10]. A compound is suitable to be a candidate biomarker if is stable in wastewater, human-specific and excreted in urine or

feces constantly, present in high concentrations in sewage water, not absorbable to particulate matter, and easily, quickly and safely determinable in wastewater [38, 39]. Therefore, finding an appropriate biomarker has become a great challenge. This approach has been used for legalized drugs, such as nicotine, caffeine and alcohol that provide very specific data on their use in society and for a few biomarkers that provide data on exposure/effects. A recent review [19] has outlined potential wastewater biomarkers of exposure or effect that could be used for future applications associated with lifestyle and wellbeing studies. **Table 1.2** summarizes the currently proposed biomarkers in wastewater, and **Table 1.3** outlines the state-of-art of their analytical determination in wastewater for WBE.

Specific human biomarkers were first proposed to estimate the tributary population to the wastewater treatment plant. The simplest methods to estimate population parameters for sewage epidemiology are based on common census (*de jure* population) or WWTP design capacity. However, these methods are not always adequate because the administrative regions may not coincide with geographic catchments of WWTP or the population census can be outdated and do not take into account fluctuations (regular commuters, tourists and demographic changes) [5, 53]. Then, alternatively, population size has been estimated using hydrochemical parameters as biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen (N) and phosphorous (P), one inhabitant is equivalent with 59 g day^{-1} BOD, 128 g day^{-1} COD, 12.5 g day^{-1} N and 1.7 g day^{-1} P [72-74]. The disadvantage of these estimations is that the biomarkers are not human-specific and could be influenced by multiple sources that contribute to these loads within sewers (food waste, domestic or/and industrial wastewater [3, 5]. Recently and due to the advanced LC-MS technics, other studies proposed to measure human-specific markers including parent compounds,

excreted metabolites or endogenous human compounds. Acesulfame, atenolol, caffeine and its metabolites, carbamazepine, furosemide, gabapentine, hydrochlorothiazide, ibuprofen, iopromide, naproxen, norfloxacine, paracetamol, salicylic acid, creatinine, nicotine and its metabolites, codeine, cortisol, androstenedione, and 5-hydroxyindoleacetic acid (5-HIAA) have been proposed [38, 39, 54, 55, 59-61]. Depending on their concentration in water, these and any of the other biomarkers that will be mentioned below could be determined by direct injection [38, 59-61] liquid-liquid extraction (LLE) [38], or SPE using either HLB or mixed mode phases [38, 39, 54, 55, 61]. LC-MS/MS or UHPLC-MS/MS methods using QqQ mass analyzers and switching between positive and negative ionization mode complete the analytical overview for these compounds. However, stability issues raised in relation to creatinine, cortisol and androstenedione [38, 59, 60]. Medication use is dependent on the preference of clinicians, on socioeconomic and other factors, and varies internationally [38]. A combination of compounds as reported in many of the studies may be more reliable than using a single population biomarker [38, 39, 54, 55, 61].

Ethyl sulfate (EtS) is excreted in urine as a minor metabolite (0.010-0.016% on molar basis) after intake of alcoholic beverages, being a convenient biomarker for ethanol tracing after its determination in sewage. Analytical methods are based on direct injection and LC–MS/MS exploiting ionic exchange mechanisms because EtS is poorly retained on conventional C_8 and C_{18} reverse phase chromatographic columns [62]. Dihexylammonium acetate (DHAAc) [41, 62, 63], dibutylammonium acetate (DBAAc) [63, 66], tetrabutylammonium bromide (TBAB) [64, 65] or trimethylamine and formic acid have been proposed as ion-pair added to either the mobile phase or the sample. However, recent studies utilize a trifunctional C_{18} alkyl ligand bonded at density that promotes polar compounds retention eliminating the need to add ion pairs [40, 68, 69].

The major tobacco alkaloid, nicotine, was targeted in wastewater analysis as population size and smokers biomarkers. The urinary metabolites, cotinine and trans-3'-hydroxycotinine, are the preferred markers because they are human specific [40, 41, 70]. As nicotine can come from nicotine gum and patches, two alkaloids – anabasine and anatabine –, which are specific to dried tobacco, have also been assessed as biomarkers for tobacco consumption in wastewater [70]. Caffeine and its metabolites, 1-methylxanthine, 7-methylxanthine and 1,7-dimethylxanthine have been determined both as population markers and to determine their consumption [39, 41]. Caffeine and its metabolites were confirmed as good qualitative biomarkers, but additional information is needed on the caffeine metabolism in relation to the multiple sources of its main metabolites.

Human urinary metabolites of the major classes of pesticides (triazines, organophosphorus and pyrethroids) were measured in urban wastewater as biomarkers of population exposure [57, 58]. Triazine and pyrethroids metabolites were extracted by SPE. However, the highly polar alkyl phosphates were poorly recovered on different SPE cartridges, so direct injection into the LC-MS/MS system was tested and adopted. Typical chromatograms of some of these biomarkers in raw wastewater and analytical standards are presented in **Fig. 1.5**. The frequency of detection and abundance of the metabolites were in line with the profiles reported in human urine. This novel method can be a valuable tool to obtain objective and direct information on the "real" levels of exposure of a specific population to pesticides and can provide additional information for human biomonitoring studies.

There are a number of works that determine simultaneously a large number of artificial sweeteners in wastewater, even though they have not been used in WBE yet. They present good characteristics, e.g. sucralose has high stability under heat and over a

broad range of pH. Acesulfame has been included as one of the potentials biomarkers for population. In addition to the reverse phase, HILIC silica-based columns have also been used, and the retention mechanism of the analytes seemed to be partition to the water layer as well as hydrogen bonding, mainly for dipeptide retention [50].

Until now, few WBE studies have explored the association between the chemical consumption measured in a population and any health impacts or environmental health factors. Isoprostanes have been proposed as suitable biomarkers of oxidative stress in a range of organisms including humans, fish, chickens, bivalves, seals and rodents [75]. Ryu et al. (2015) [56] quantitatively analysed for the first time reliable oxidative stress biomarker, 8-iso-prostaglandin $F_{2\alpha}$ in wastewater using an analytical method consisting of liquid LC-HR-MS coupled to immunoaffinity clean-up.

Fattore et al., (2016) [71] reported the association between asthma and outdoor PM10 and PM2.5 levels by using the levels of salbutamol in wastewater as an indicator of the occurrence of asthma. Such findings provided direct evidence of the effect of outdoor ambient air pollution on asthma, which is usually difficult to obtain by other methods. Health biomarkers can be pharmaceuticals and their metabolites. Phung et al. (2017) [51] utilised a unique WBE data set to investigate the association between the ambient temperature and the levels of eight pharmaceuticals and personal care products measured in wastewater for more than a year. The outcome was consequently used to select good candidate biomarkers for health impact of ambient temperature for future WBE studies. The results indicated that an increase of 1º C in average temperature is associated with decrease for atenolol as well as increase for acesulfame, and increase at for naproxen. No significant association was observed between temperature and the caffeine, carbamazepine, codeine, hydrochlorothiazide, and salicylic. The hypothesis is that consumption of sweetened drinks, risk of cardiovascular diseases and pains are

associated with changes in ambient temperature. However, these statements requires further confirmation.

3. LC-MS to wide-scope screening and identification of unknown drugs of abuse and transformation products

New strategies have been reported for "*suspected screening*" and "*non-target analysis*" in environmental samples using LC coupled to QqTOF MS, QTRAP-Orbitrap and Q-orbitrap thanks to their ability to provide the most probable empirical formula as well as MS/MS information. The easiest way to work (reported in **Table S1.1**) is the "*suspected screening*", "*non-target screening*" or "*wide-scope screening*". The compounds are identified extracting the exact *m/z* ion chromatograms with a narrow window against a database that contains in addition to the empirical formula information on the isotopic abundance, the number of double bonds, purity score of the product ion mass spectra to confirm the identify without the need of analytical standards. The hardest way to operate is the "*non-target"* analysis or "*unknown identification*" that implies the recognition of compounds that remains unknown after target and suspected screening. As **Table 1.4** shows the few studies dealt with any type of non-target methodology to identify metabolites and/or degradation products and even NPS. DDA and DIA have been indistinctly used for this purpose. Special mention deserves the QTRAP-Orbitrap that combines orbitrap with a linear ion-trap, allowing to get HR-MS in the orbitrap and nominal MS/MS in the QTRAP among other fragmentation options.

Non-target screening becomes a challenging task, but, for metabolites, degradation or transformation products (since now TPs), further information of the parent compound (e.g., molecular formula, MS/MS spectrum, t_R and other physico-chemical data)

contributes to ranking of possible structures and simplifies the identification process. The TPs or unknown identification involves the establishment of some criteria, such as the accuracy of the molecular ion (e.g., mass error < 5 ppm, dependent on the mass accuracy) and the characteristic fragment ions in MS/MS mode (purity score ≥ 65 recommended). Matching most probable empirical formula to chemical structure is aided by exploration of databases, such as ChemSpider, PubChem, or NIST. Thereby, information on the parent compound (e.g., molecular formula, substructures) quoted the databases search and enabled to propose possible structures that are ranking comparing the MS/MS spectra to in-silico mass spectral fragmentation or to spectra in libraries. There are a few databases with LC mass spectra, e.g.: MassBank (http://massbank.ufz.de/MassBank/); and, MetLin (http://metlin.scripps.edu/index.php) that are very useful. This strategy was already successfully applied to WBE [25, 81-83].

The problem with HR-MS is how to evaluate the massive quantities of data generated. For this reason, post-acquisition data-processing tools are necessary; computer-aided techniques provide rapid, accurate and efficient data mining. There is a number of open-source and commercial software options for non-target screening based on the comparison of blanks (controls, or controlled positive) to the treated, problem or unknown samples that select only those compounds that make a difference between both groups of samples. These tools commonly are able to make principal component analysis (m/z vs retention time). There are also software that recognize a parent compound using information on common metabolism or transformation reaction for contaminants in wastewater. This strategy was exploited to identify THC-COOH metabolites and TPs of 42 illicit drugs and NPS. **Fig. 1.6** shows MS and MS/MS of a potential ephedrine TP [$C_9H_{13}NO_2$] identified by the software resulting from the demethylation and oxidation. This compound matches 1-(3-hydroxyphenyl)-2-

(methylamino) ethanone or phenylephrine, a legal compound used as nasal decongestant. This was confirmed by a search of the molecular formula in the METLIN database.

An important help to predict them is the application of a number of "*in-silico*" based tools. Reid et al. (2014) [76] applied SMARTCyp from University of Minnesota Pathway Prediction System (UM-PPS: http://eawag-bbd.ethz.ch) to identify biomarkers of NPS (including transformation products) [76]. These models do not guarantee the formation of a given metabolite or biotransformation product. So, they must be coupled to an extensive non-targeted screening including common fragment searches to identify related compounds that share structural elements and mass-defect filtering (the majority of metabolites of a compound have a mass defect of within 50 mDa of that of the parent). Furthermore, the difficulty is exacerbated by the low concentration in wastewater often below the lower limit of detection. Reid et al. (2014) [76] also proposed as primary alternative the collection and analysis of wastewater from pissoirs to identify biomarkers of NPS prior to search them in wastewaters. Alternatively, Lai et al. (2015) [78] addressed this problem by a careful design of in vitro metabolism experiments using subcellular liver fractions to establish a list of specific in vitro human metabolites for phenethylamine-based designed drugs identification in wastewater. In other study, Mardal et al. (2016) [84] evaluate the in vivo and in vitro metabolism as well as microbial biotransformation of excretion products and unchanged 3-fluorophenmetrazin. Thanks to these study, the proposed strategy for WBE is its quantitative determination unchanged together with qualitative verification of a number of selected metabolites to verify consumption and rule out discharge.

Therefore, the combined collection of these tools and alternative data-sources provide an excellent framework which can be used to maximize the chances of success

in identifying and detecting biomarkers of NPS, new transformation products or unknowns in wastewater.

4. Conclusion and future trends

WBE has become an essential and well-establish tool to determine the consumption of illicit drugs by populations. The high numbers of studies dealing with these estimations in wastewater through LC-MS and applying WBE that have been conducted to date give a good indication of the relevance of this approach for society, researchers and authorities. LC-MS is now the reference analytical technique because of the compatibility of aqueous samples with RP-LC system and the ability of LC-MS to detected most of illicit drugs and related-human biomarkers. This reason, as well as the ability of LC with triple quadrupole or linear ion trap mass analyzers to quantify selected compounds in target methodologies with outstanding sensitivity and selectivity makes LC-tandem-MS one of the most powerful analytical tools available in WBE.

Nowadays, this approach is being expanded to other biomarkers, consumptions and exposures, such as new psychoactive substances, alcohol, tobacco, pesticides. However, these studies pointed out that using targeting approaches, parent compounds or expected biomarkers are not found. In this sense, the replacement of the conventional LC-MS by LC-HRMS according to the accurate m/z values of the ions is highly promising due to its potential to perform suspected screening against a database containing large list of compounds as well as to detect and identify unknown compounds. Furthermore, from the perspective of enlarge the goal of WBE, it is of interest to extend this research to new biomarkers, metabolites and transformation products, which in many occasions are still unknown.

The potential of WBE will be expanded to other aspects of public health. Results from environmental degradation, metabolism, toxicology and epidemiology studies could present more targets for method developing, such as biomarkers of health, disease, life style, etc. Therefore, the development of novel analytical methods for the detection of specific and long term biomarkers will provide new strategies for BWE development and will be crucial to large scale metabolic phenotyping for health monitoring through the early detection of biomarkers in wastewater.

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Table 1.1. New psychoactive substances searched in wastewater using LC-MS

Classes	Substances	Ref.
Synthetic cannabinoids	• 1-(5-fluoropentyl)-3-(2-iodobenzoyl)indole (**AM-694**) • 1-(5-fluoropentyl)-3-(1-naphthoyl)indole (**AM-2201**) • (2-iodophenyl)[1-[(1-methyl-2-piperidinyl)methyl]-1H-indol-3-yl]-methanone (**AM-2233**) • naphthalen-1-yl-(4-pentoxynaphthalen-1-yl)methanone (**CB-13** or **CRA-13**) • (2-Methyl-1-pentyl-1H-indol-3-yl)(1-naphthyl)methanone (**JWH-007**) • (1-Butyl-2-methyl-1H-indol-3-yl)(1-naphthyl)methanone (**JWH-016**) • 1-Naphthyl (1-pentyl-1H-indol-3-yl) methanone (**JWH-018**) • (1-hexyl-1H-indol-3-yl)(naphthalen-1-yl)methanone (**JWH-019**) • 1-Naphthyl (1-butyl-1H-indol-3-yl) methanone (**JWH-073**) • (4-methoxynaphthalen-1-yl)-(1-pentylindol-3-yl)methanone (**JWH-081**) • J (4-methoxynaphthalen-1-yl)-(2-methyl-1-pentylindol-3-yl)methanone (**JWH-098**) • (4-methylnaphthalen-1-yl)-(1-pentylindol-3-yl)methanone (**JWH-122**) • (1-hexyl-5-phenylpyrrol-3-yl)-naphthalen-1-ylmethanone (**JWH-147**) • 2-(2-Chlorophenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone (**JWH-203**) • pentyl-1H-indol-3-yl)methanone (**JWH-210**) • (4-Methyl-1-naphthyl) (1-pentyl-1H-indol-3yl)methanone (**JWH-122**) • 2-(2-Methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl) ethanone (**JWH-250**) • 2-(2-methylphenyl)-1-(1-pentylindol-3-yl)ethanone (**JWH-251**) • 2-(3-Methoxyphenyl)-1-(1-pentyl-1H-indol-3-yl)ethanone; 2-(3-methoxyphenyl)-1-(1-pentylindol-3-yl)ethanone (**JWH-302**) • (5-(2-fluorophenyl)-1-pentyl-1H-pyrrol-3-yl)(naphthalen-1-yl)methanone (**JWH-307**) • 1-Pentyl-3-(4-chloro-1-naphthoyl)indole (**JWH-398**) • 1-pentyl-3-(4-methoxybenzoyl)indole (**RCS-4**) • 1-(2-cyclohexylethyl)-3-(2-methoxyphenylacetyl)indole (**RCS-8**)	[24-26]
Synthetic cathinones	• Ephedrine **(EPH)** • Dibuthylone **(bk MMBDB)** • Butylone (**BUTL**) • Cathinone **(CATH)** • Ephedrone **(EPHED)** • Mephedrone **(MEP)** • Methcathinone (**METC**) • Methylenedioxypyrovalerone (**MDPV**) • Methylone (**METL**) • α-pyrrolidinovalerophenone (**α-PVP**) • α-pyrrolidinopropiophenone (**PPP**) • 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (**MDPPP**) • 4-methyl- α-pyrrolidinopropiophenone (**4-MePPP**) • 4′-methyl-α-pyrrolidinohexanophenone (**4′MePHP**) • 4-methyl-α-pyrrolidinobutirophenone (**MPBP**) • N,N-dimethylcathinone (**DCAT**) • β-ethyl-methcathinone [pentedrone (**PENT**) • Ethylone (**ETONE**) • Naphyrone (**NAPH**) • 1-Naphyrone **(1NAPH)** • Ethcathinone (**ETHC**) • Methedrone (**METH**) • 4-fluoromethcathinone (**4-FMC**) • 3,4-dimethylmethcathinone (**3,4-DMMC**) • 4-methylethcathinone (**4-MEC**) • Buphedrone (**BUPH**) • Pentedrone **(PEN)** • Pentylone (**PENTL**)	[24-36]
Phenethylamines	• 2-(4-chloro-2,5-dimethoxyphenyl)-N-(2-methoxybenzyl)ethanamine (**25-C-NBOMe**) • 2-(4-iodo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine (**25-I-NBOMe**) • 2-(4-bromo-2,3,6,7-tetrahydrofuro[2,3-f][1]benzofuran-8-yl)-N-[(2-methoxyphenyl) methyl]ethanamine (**25-B-NBOMe**) • 1,3-benzodioxolyl-N-methylbutanamine (**MBDB**) • 4-Fluoroamphetamine **(4-FLU)** • Methamphetamine (**MAMP**) • 3,4-Methylenedioxyamphetamine (**MDA**) • 3,4-Methylenedioxymethamphetamine (**MDMA**) • 3,4-Methylenedioxyethylamphetamine (**MDEA**) • 4-bromo--2,5-dimethoxyphenethylamine (**2-CB**) • Methiopropamine (**MPA**)	[24, 25, 30, 32-35]

	• 4-methoxymethamphetamine (**PMMA**) • 4-methoxyamphetamine (**PMA**) • Ethylamphetamine (**ETAMINE**) • 3,4-methylenedioxypyrovalerone (**MDPV**)	
Piperazines	• 1-Benzylpiperazine (**BZP**) • Trifluoromethylphenylpiperazine (**TFMPP**) • 1-(3-Chlorophenyl)piperazine (**mCPP**)	[24, 26, 28, 33-35]
Ketamine and phencyclidine-type substances	• Ketamine **(KET)** • Norketamine **(NKET)** • Dehydronorketamine • 4-methoxy phencyclidine **(4-MeO-PCP)**	[24, 25, 27]
Tryptamines	• 4-acetoxy-N,N-dimethyltryptamine **(4-AcO-DIPT)** • Bufotenine **(BUF)**	[24]
Other	• 1-methyl-4-phenyl-4-propionoxypiperidine **(MPPP)** • Methoxetamine **(MXE)** • Methylphenidate **(MEPHEN)**	[24, 26]

Table 1.2. Examples of Existing and Potential Wastewater Biomarkers of Application in WBE

Indicator of	Biomarker	Determination in wastewater	WBE	Ref.
Life style				
Alcohol	Ethyl sulfate (EtS)	LC-MS	✓ Yes	[37]
Tobacco	Nicotine, cotinine, 3-hydroxycotinine, anabasine, anatabine	LC-MS	✓ Yes	[40-46]
Illicit drugs	Cocainics, opioids, amphetaminics, etc.	LC-MS	✓ Yes	[11, 18, 19, 21] **Table S1.1**
Anabolic steroids	Synthetic steroids and its metabolites	LC-MS	✓ Yes Little	[38]
Diet				
Artificial sweeteners	Saccharin, cyclamate, aspartame, acesulfame, neohesperidin dihydrochalcone, sucralose, stevioside, glycyrrhizic acid	LC-MS	Only acesulfame	[47-51]
Urinary sugars	Sucralose	✖ NO	✖ NO	[10]
Soya	Phytoestrogens: isoflavones, enterolignans and coumestrol	LC-MS	✓ Yes Little	[52]
Fruits and vegetables	Flavonoids	✖ NO	✖ NO	[10]
Stimulant beverages	Caffeine	LC-MS	✓ Yes Further studies needed	[39, 41, 53]
Meat	taurine, 1-methylhistidine, 3-methylhistidine	✖ NO	✖ NO	[10]
Health				
Pharmaceuticals	Atenolol, carbamazepine, codeine furosemide, gabapentine, hydrochlorothiazide, ibuprofen, iopromide, naproxen, norfloxacine, paracetamol, salicidic acid	LC-MS	✓ Yes	[5, 38, 54, 55]
Oxidative stress	F2-isoprostanes, 8-hydroxydeoxyguanosine	LC-MS	✓ Yes Little	[56]
Pregnancy	ss-human chorionic gonadotropin	✖ NO	✖ NO	[10]
Allergy	antihistamines	✖ NO	✖ NO	[10]
Cancer	R-Fetoprotein (AFP; cancer), carcinoembryonic antigen (CEA), PSA, CA125, CA15.3, CA19.9, immunoglobulins, chroriogonadotropin (hCG)	✖ NO	✖ NO	[10]
Endogenous compounds	Creatinine, 5-HIIA	LC-MS	✓ Yes Little	[38]
Exposure				
Pesticides	Urine biomarkers of triazines, pyrethroids and organophosphates	LC-MS	✓ Yes	[57, 58]
Parabens (PB)	Methyl-PB; ethyl-PB; propyl-PB; buthyl-PB	LC-MS	✖ NO	[19]
Mycotoxins	Deoxynivalenol, beauvericin, 3-Acetyldeoxynivalenol, nivalenol, zearalenone, α-zearalenol and β-zearalenol	LC-MS	✖ NO	[19]
UV-Filters	Benzophenone derivatives, p-aminobenzoic acid derivatives, camphor derivatives, benzotriazole derivatives, salicylate derivatives, benzimidazole derivatives, triazine derivatives, cinnamate derivatives, crylene derivatives, and dibenzoylmethane derivatives	LC-MS	✖ NO	[19]
Flame retardants	Urinary metabolites of brominated flame retardants (BFRs) and organophosphorus flame retardants (PFRs)	GC-MS/LC-MS Urinary metabolites not reported in wastewater yet	✖ NO	[19]
Plasticizers	Metabolites of phthalates, adipates, and di-isononyl cyclohexane-1,2-dicarboxylate	✖ NO	✖ NO	[19]

Table 1.3. Overview on studies evaluating biomarkers of consumption/exposure/effects by LC-MS in WBE

	Biomarker	Extraction	LC		MS detection	Sensitivity LODs (ng L^{-1})	Ref.
			Column	Mobile Phase			
Population size	Caffeine and its major metabolites: Paraxanthine, 1-methylxanthine, 7-methylxantin Nicotine and its major metabolites Cotinine -3'-hydroxycotinine	SPE with Oasis HLB cartridges and elution with MeOH	X-Terra C18 (100 x 1 mm, 3 µm)	Gradient 5 mM AmAc in water and acetonitrile at 0.07 $mLmin^{-1}$	QqQ-MS/MS, ESI (+) SRM IS: caffeine-^{13}C3; 1,7-dimethyluric acid-d3	3.6 – 28.1	[39]
	Atenolol, Codeine, Caffeine, Hydrochlorthiazide, Acesulfame, Salicylic acid, Carbamazepine, Naproxen	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH automated robot for preparation	Kinetics C18 (100X 3 mm, 2.6 µm)	Gradient water-acetonitrile both with 0.1 % FA	QqQ-MS/MS, ESI (+)/ESI (-) SRM IS: c	Not specified	[53]
	Acesulfame, Atenolol, Caffeine, Carbamazepine, Furosemide, Gabapentine, Hydrochlorothiazide, Ibuprofen, Iopromide Naproxen, Norfloxacine, Paracetamol, Salicidic acid	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH	Luna C-18 (2) (150 × 3 mm, 3 µm, 100 Å,) at 35 ºC	Gradient water-acetonitrile both with 0.1 % FA	QqQ-MS/MS, ESI (+) and ESI (-) (acesulfame, ibuprofen, hydrochlorothiazide, salicidic acid and naproxen) SRM IS: acesulfame-d4, caffeine-d3, hydrochlorothizide-C13d2	Not specified	[54]
	Creatinine	DI	Luna C18 (150×4.6 mm, 5 µm)	Isocratic: 10mM AmAc in 5% MeOH	QqQ-MS/MS, ESI (+) SRM IS: Not used	50000	[59]
	Creatinine	DI	Phenomenex Security Guard SCX cartridge (4.0 x 3.0 mm)	Gradient of water 0.25 % AcH and MeOH at 0.6 $mLmin^{-1}$	QqQ-MS/MS, APCI (+) SRM IS: creatinine-d3	3000	[60]
	Atenolol, Codeine, Caffeine, Hydrochlorthiazide, Acesulfame, Salicylic acid, Carbamazepine, Naproxen	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH automated injector	Kinetics C18 (100 × 3 mm, 2.6 µm)	Gradient water-acetonitrile both with 0.1 % FA at 0.7 $mLmin^{-1}$	QqQ-MS/MS, ESI switching between (+/-) SRM IS: atenolol-D7, codeine-D3, caffeine-13C, hydrochlorothiazide-13C-D2, acesulfame-D4, acetyl sulfamethoxazole-D4 and carbamazepine-D10	10	[55]
	Creatinine	DI-LVI (Creatinine) Adjusted pH = 2 (HCl) and extract with ethyl	Luna C18 (150 × 4.6 mm,5 µm)	Gradient 20 mM AmF (pH 9) and MeOH at 0.5 mL min^{-1}	QqQ-MS/MS, ESI (+) SRM IS: creatinine-d3	3000	[38]

	Biomarker	Extraction	LC		MS detection	Sensitivity LODs (ng L^{-1})	Ref.
			Column	Mobile Phase			
	Cortisol Androstenedione 5-HIIA	acetate (5-HIAA) Adjusted at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH (The others)	ZORBAX Eclipse XDB-C18 (50 × 4.6 mm, 1.8 µm)	Gradient water 0.1 % FA and MeOHl at 0.5 $mLmin^{-1}$	QqQ-MS/MS ESI (+) SRM IS: 5-HIAA-d5, androstenolone-d5	30	[38]
	Cotinine		Luna™ pentafluorophenyl (PFP(2)) column (50 × 4.6 mm, 3 µm, 100 Å)	Gradient water 0.1 % FA and MeOHl at 0.5 $mLmin^{-1}$	QqQ-MS/MS ESI (+) SRM IS: Not used	5	[38]
	5-HIAA, atenolol, caffeine, carbamazepine, codeine, creatinine, naproxen, cotinine, hydroxycotinine	Dilute (50:50, v/v) with distilled water and DI or SPE with Strata-X 33 µm cartridges and elution with MeOH	Kinetex C18 (50 x 2.11 mm, 1.7 µm)	Gradient water and MeOH both 1 % FA at 0.2 $mLmin^{-1}$	QqQ-MS/MS ESI (+) SRM IS: not used	500-1500 (DI) 4-309 (SPE)	[61]
	Acesulfame, salicylic acid, hydrochlorothiazide			Gradient water and meOH both 2 mM NH_4F at 0.2 mL min^{-1}	QqQ-MS/MS ESI (-) SRM IS: not used		
Alcohol intake	Ethyl sulfate (EtS) Ethyl glucuronide (EtG)	Wastewater centrifuged DI	Acquity UPLC Bridged-Ethyl Hybrid C8 column (1.7 µm, 50 × 2.0 mm) at 50°C	Dihexylammonium acetate (7 mM) in a MeOH-water gradient at 0.6 $mLmin^{-1}$	QqQ-MS/MS ESI (-) SRM IS: EtS-D5; EtG-D5	EtS --- EtG ---	[41, 62, 63]
	EtS	Wastewater centrifuged Add 50 mM tetrabutylamonium bromide to the sample. DI	Synergi 4 µm Fusion-RP column (100 mm × 2.0 mm) at 45 ºC	Gradient of MeOH water both with 0.1 % of FA at 0.4 mL min^{-1} at 0.4 $mLmin^{-1}$	QqQ-MS/MS and QqTOF ESI (-) SRM and 20 mDa XIC IS: EtS-D5	300	[64, 65]
	EtS	Wastewater centrifuged DI	STAR RP-18 end-capped column (125 mm × 2.0 mm, particle size 5 µm)	dibutylammonium acetate (5 mM) in a MeOH water gradient at 0.3 $mLmin^{-1}$	QqLIT -MS/MS ESI (-) SRM IS: EtS-D5	70	[63, 66]
	EtS	Wastewater centrifuged Add 0.5 M tributylamine and .1% of FA to the sample. DI	Kinetex C18 (1.7 µm, 100 Å, 50 × 2.10 mm) at 30 ºC	Isocratic: 90% 0.1% FA in water) and 10% eluent 0.1% FA in MeOH at 0.3 $mLmin^{-1}$	QqQ-MS/MS, ESI (-) SRM IS: EtS-D5	300	[67]
	EtS	Wastewater centrifuged DI	Atlantis T3 column (150 mm × 2.1 mm, 3 µm) at 30 °C	Gradient 0.1 % FA in water and acetonitrile at 0.3 $mLmin^{-1}$	QqQ-MS/MS, ESI (-) SRM IS: EtS-D5	1500	[68]
	EtS	Wastewater centrifuged DI	Atlantis T3 column (150 × 2.1 mm, 3 µm) at 20 °C	Gradient 0.1 % FA in water and acetonitrile at at 0.18 $mLmin^{-1}$	QqQ-MS/MS, ESI (-) SRM IS: EtS-D5	1500	[40, 69]
Tobacco use	Nicotine Cotinine 3-OH cotinine	SPE with Oasis HLB cartridges and elution with MeOH	X-Terra C18 (100 x 1 mm, 3 µm)	Gradient 5 mM AmAc in wáter and acetonitrile at 0.07 $mLmin^{-1}$	QqQ-MS/MS, ESI (+) SRM IS: cotinine-D3, nicotine-D3	0.43 – 11.5	[40, 41]

	Biomarker	Extraction	LC		MS detection	Sensitivity LODs (ng L^{-1})	Ref.
			Column	Mobile Phase			
	Cotinine, anabasine, anatabine	Adjusted to pH 4.5–5, SPE using UCT-XRADH506 Elution with CH_2Cl_2: isopropanol: NH_3 (78%:18%:4 %)	Supelco HS F5 (150 X 4 mm, 5 µm)	Gradient acetonitrile and water both with 10 mM AmF, 0.1% FA and 0.1% THF	QqTOF-MS/MS, ESI (+) set for unit resolution and high sensitivity SRM IS: anabasine D4, anatabine D4, nicotine D4, cotinine D3	20-400	[42]
	Cotinine	In-line SPE C18 column (Hypersil Gold, 20 x 2.1 mm , 12 µm)	Cogent bidentate (50 x 2.1 mm, 3 µm)	Gradient acetonitrile and water both, 0.1 % FA at 0.3-0.4 µm.	Q-Orbitrap ESI+, full MS and target MS/MS QqQ-MS/MS, ESI +, SRM IS: cotinine D3	5-10	[43, 44]
	Nicotine Cotinine OH-Cotinine	SPE with reverse-phase cartridges and elution with MeOH	Waters Symmetry Shield C18 (150 × 2.1 mm,3.5 µm)	Gradient water and MeOH both with 5 mM AmAc at 0.4 mL/min	QqQ-MS/MS, ESI +, SRM IS: nicotineD3, cotinineD3, OH-cotinineD3	12-20	[70]
	Cotinine	Water at pH 6, SPE with Oasis HLB cartridges and elution with MeOH	Luna HILIC 200 Å (150 × 3.00mm, 5 µm) at 35 ºC	Gradient water with 5 mM AmF and acetonitrile at 0.3 mL/min	QqQ-MS/MS, ESI +, SRM IS: cotinine D3	14.9	[46]
Exposure to pesticides	Urine biomarkers of Triazine Pyrethroids Organophosphates	SPE with Oasis HLB cartridges and elution with MeOH DI	XSELECT™ CSH™C18 (2.1×100 mm, 2.5 µm)	Gradiente water 0.1 % AA and acetonitrile at 0.18 mL/min	QqQ-MS/MS, ESI +/-, SRM IS: 3-PBA-C6, TCPY-C3	SPE: 1–15 DI: 40–800	[57]
	Pyrethrins (3-PBA, cis- and trans-DCCA)	Automated SPE with Oasis HLB cartridges and elution with MeOH	XSELECT™ CSH™C18 (2.1×100 mm, 2.5 µm)	Gradiente water 0.1 % AA and acetonitrile at 0.18 mL/min	QqQ-MS/MS, ESI +, SRM IS: Not used	5-15	[58]
Lifestyle and habits	Caffeine, p-xanthine 1-methylxanthine, 7-methylxanthine, 1,7-dimethyluric acid, 1-methyluric acid	SPE with Oasis HLB cartridges and elution with MeOH	X-Terra C18 (100 x 1 mm, 3 µm)	Gradient 5 mM AmAc in wáter and acetonitrile at 0.07 $mLmin^{-1}$	QqQ-MS/MS, ESI (+) SRM IS: caffeine-$^{13}C3$; 1,7-dimethyluric acid-d3	3.6 – 28.1	[41]
	Artificial sweeteners Acesulfame Cyclamate Saccharin	Filtration DI	Dionex IONPAC® AS20 analytical column (2 x 250 mm)	Gradient of potassium hydroxide at different concentrations at a 0.2 $mLmin^{-1}$	QqQ-MS/MS, ESI (-) SRM IS: sucralose-d6, acesulfame-d4, saccharin-$^{13}C6$ and cyclamic acid-d11,	8 - 21	[47]
	saccharin, cyclamate, aspartame, acesulfame, neohesperidin dihydrochalcone, sucralose, stevioside. glycyrrhizic acid	SPE with Oasis HLB cartridges and elution with MeOH	Ascentis Express RP-amide (100 × 2.1 mm, 2.7 µm)	Gradient water 0.1% FA and acetonitrile at 0.4 $mLmin^{-1}$	QqQ-MS/MS, ESI (-) SRM IS: Not used	500-600	[48]
	Acesulfame K, aspartame, saccharin Na, and sucralose	Adjust to pH 2 SPE with Oasis HLB cartridges and elution with 40 % of acetonitrile in water	Eclipse XDB-C18 (150 x 2.1-mm, 3.5-µm)	Isocratic 5 mM of dibutylammonium acetate and 80 % acetonitrile in water at 0.4 $mLmin^{-1}$	LC-single quadrupole-MS SIM IS: Not used	0.015-23	[49]

	Biomarker	Extraction	LC		MS detection	Sensitivity LODs (ng L^{-1})	Ref.
			Column	Mobile Phase			
	Aspartame, alitame, neotame, acesulfame, saccharin, cyclamate , sucralose, neohesperidin dihydrochalcone	Adjust to pH 6 Strata X cartridges (similar to HLB) and elution with MeOH	XBridge HILIC (2.1 x 150 mm, 3.5 µm) and Kinetex HILIC (2.1 x 100 mm, 3.6 µm)	Isocratic 5mM ammonium formate pH 3.5–MeOHl–acetonitrile (15/10/75 v/v/v) at 0.1 $mLmin^{-1}$	LTQ-MS^2,MS^3,MS^4, ESI (-) SRM IS: acesulfame-d4, sucralose-d6, aspartame-d3	240-4400	[50]
	Phytoestrogens (isoflavones, enterolignans and coumestrol)	Adjust to pH 5 SPE with Oasis HLB cartridges and elution with MeOH	XTerra MS C18 column (2.1 x 150 mm, 3.5 µm at 26 ºC	Gradient water 0.1% FA and acetonitrile at 0.2 $mLmin^{-1}$	LTQ-MS^2,MS^3,MS^4, ESI (-) SRM IS: genistein-d4	0.5-3	[52]
Health	F2-Isoprostaglandin isomer	Wastewater enzymatic hydrolysis with β-glucuronidase. Analytes were isolated on immunoaffinity columns	Acquity UPLC HSS C18 column (1.8 µm, 150 × 2.1 mm) at 50 ºC	Gradient of water and acetonitrile both 0.01% acetic acid at 0.4 mL min^{-1}.	UHPLC-QqTOF-MS MS^E (m/z 50–800)	0.3	[56]
	Salbutamol	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH	Luna C8(2) (50 × 2 mm, 3 µm) Or for calculate EF_{rel} Astec Chirobiotic C18 (250 × 2.1 mm, 5 µm)	Gradient water 0.1% FA and acetonitrile at 0.2 $mLmin^{-1}$ Or 99.95% MeOH with 4 mM AmAc and 0.005% FA	QqQ-MS/MS, ESI (+) SRM IS: Salbutamol-D3	0.3	[71]
	Atenolol, acesulfame, naproxene from study [53]	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH automated robot for preparation	Kinetics C18 (100X 3 mm, 2.6 µm)	Gradient water-acetonitrile both with 0.1 % FA	QqQ-MS/MS, ESI (+)/ESI (-) SRM IS: c	Not specified	[51]

3-PBA: 3-Phenoxybenzoic acid; 5-HIAA: 5-hydroxyindoleacetic acid; AA: acetic acid; AmAc: ammonium acetate; AmF: ammonium formate; DI: direct injection; FA: formic acid; IS: internal standard; LOD: limit of detection; LTQ: linear ion trap; LVI: large volume injection; MeOH: methanol; SCX: strong cation exchange; SIM: selected ion monitoring; SRM: selected reaction monitoring; TCPY: 3,5,6-trichloro-2-pyridino

Table 1.4. Overview on studies applying suspected screening and non-target identification by LC-HR-MS in WBE

Purpose	Target compounds	Extraction	MS detection	Strategies	Ref.
LC-QqTOF-MS					
Biomarkers predicted "*via in-silico*" metabolism, biotransformation and sorption models and subsequent detection in wastewater	NPS and their metabolites	LLE dicholormethane-isopropanol-ethyl acetate mixture (1: 1: 3 v/v/v).	**DIA**: QqTOF-MS^E (50 – 800 m/z) PI mode using CE ramp (15 – 15 V) **Screening** and identification using a library of 1000 compounds (t_R and precursor, product ion mass accuracy)	**Additional strategies**: - common-fragment searching - mass-defect filtering **Aids:** - in-silico models predict transformation	[76]
THC-COOH as cannabis biomarker in waters and on the formation of possible TPs	THC-COOH and its TPs	SPE using Oasis HLB and elution with MeOH	**DIA:** QqTOF-MS^E (50 – 800 m/z) PI and NI modes using CE ramp (15 – 15 V) **Target analysis** and **Screening** and identification using a library of 1000 compounds (t_R and precursor, product ion mass accuracy)	**Additional strategies:** - MetaboLynx XS compares experimental studies to controls **Aids:** - laboratory controlled degradation experiments to identify TPs	[77]
TPs and unknown in water	42 Drugs, NPS, TPs	SPE using Oasis HLB and elution with MeOH	**DDA:** QqTOF-MS/(MS of ions >1000 counts) (50-600 m/z) PI mode using CE 40 V **Target analysis** and **Screening** and identification using a library of 1000 compounds (t_R and precursor, product ion mass accuracy)	**Additional strategies:** - Metabolite pilot compares experimental studies to controls and identifies some patterns **Aids:** - MetLin	[24]
A set of metabolites for meaningful evaluation of NPS in wastewater	Phenethylamines	----	**DDA:** QqTOF-MS/(MS of ions >2000 counts) with ≤ 3 precursor ions/cycle (50-600 m/z) PI and NI mode using CE 10, 20 and 40eV **Identification criteria:** MS: m/z experimental≤10 ppm; MS/MS: m/z experimental≤20 ppm; isotopic pattern 70 % over predicted one; DBE match; absence of peak in control samples; t_R < parent; logical structure considering parent and reaction	**Aditional strategiess:** - knowledge of the researchers - human in vitro metabolites of the phenethylamines **Aids:** - software to predict metabolites.	[78]
LC-Q-Orbitrap					
Studies on the microbial biotransformation of NPS	Methylenedioxy pyrovalerone (MDPV)	SPE using Biotage Isolute HCX and elution with MeOH QuEChERS	**DDA** MS m/z 66.5-1000 (70000 FWHM)/ddMS^2 ACG target 2e5 (17000 FWHM), NCE 35 %, dynamic exclusion **Target** MS^2 of a list of precursor ions **Identification criteria:** interpretation of the mass shifts in their HR-MS^2 spectra in relation to the HR-	**Aids:** - the OECD guideline 314 A for incubation in wastewater - metabolites in rats and human urine	[79]

Purpose	Target compounds	Extraction	MS detection	Strategies	Ref.
			MS^2 spectrum of the parent compound and in accordance to previously published data		
Magnitude of emergent contaminant releases through target screening and metabolite identification	Illicit drugs	On-line SPE Hypersep Retain PEP column (20 × 2.1 mm × 12 µm)	**DDA** MS m/z 100-500 (35000 FWHM)/ddMS^2 AGC target 1e5 (35000 FWHM), NCE 35 % with <3 ions/cycle and dynamic exclusion **Target** MS^2 of a list of precursor ions at 35000 FWHM at 10, 25 and 35 eV **Identification criteria:** match exact masses to an empirical formula and knowledge on the type of transformation	**Aditional strategiess:** - Use a metabolite finder software (create a list of possible phase I and II reaction and their corresponding exact mass difference expected in the products.	[80]
LC-QTRAP-Orbitrap					
Target analysis and potential for retrospective analysis	Illicit drugs	SPE using Oasis HLB and elution with MeOH (authomatic) using a GX-274 ASPEC	**DDA:** MS (exact mass) and MS^n (nominal mass) (50-600 m/z at 30000 FWHM), intensity threshold and a target list. **Identification criteria:** Accurate mass of $[M+H]^+$, together with >1 nominal mass product ion and t_R	**Aids:** - confirmation of the results using the analytical standards	[81, 82]
Screening new psychoactive substances in urban wastewater	52 NPS	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH	**DDA:** MS (exact mass at 60000 FWHM) **top5** MS^n (nominal mass) (150-600 m/z), intensity threshold. **Target** MS^2 of a list of precursor ions (5 or 6) **Identification criteria:** curate mass of $[M+H]^+$, DBE and isotopical pattern together with >1 nominal mass product ion and t_R	**Aditional strategies:** - A library of MS/MS spectra of 16 synthetic cathinones and 19 synthetic cannabinoids at different collisions energy	[25]
Transformation products of drugs of abuse	Ozonation products	Water at pH <3, SPE with Oasis MCX cartridges and elution with MeOH and 2 % of NH_4OH using an automatic system to inject.	**DDA:** MS (exact mass) and MS^4 (nominal mass) (50-600 m/z), intensity threshold and a target list. **Identification criteria:** curate mass of $[M+H]^+$, DBE and isotopical pattern together with >1 nominal mass product ion and t_R	**Aditional strategies:** - ozonation at laboratory scale of water with high concentration of drugs	[83]
Identify in vivo phase I and II metabolites of the NPS	3-fluorophenmetrazin	----	**DDA** MS m/z 66.5-1000 (35000 FWHM)/ddMS^2 ACG target 2e5 (17000 FWHM), NCE 35 %, dynamic exclusion **Target** MS^2 of a list of precursor ions without dynamic exclusion **Identification criteria:** curate mass of $[M+H]^+$, DBE and isotopical pattern together with >1 nominal mass product ion and t_R	**Additional strategies:** - complementary GC-HR-MS **Aids:** - *In vivo* metabolism in humans and rats, *in vitro* contribution of CYP isoenzymes and microbial biotransformation	[84]

CE: collision energy; CYP: Cytochrome P450; DBE: double bond equivalents; DDA: data independent acquisition; DIA: data independent acquisition; FWHM: full width at half maximum; LLE: liquid-liquid extraction; NCE: normalized collision energy; NPS: new psychoactive substances; PI: positive ionization; THC-COOH: 11-nor-9-carboxy-Δ9-tetrahidrocannabinol; SPE: solid phase extraction; TPs: Transformation products; MeOH: methanol;; QuEChERS: quick, easy, cheap, effective, rugged, safe; t_R: retention time

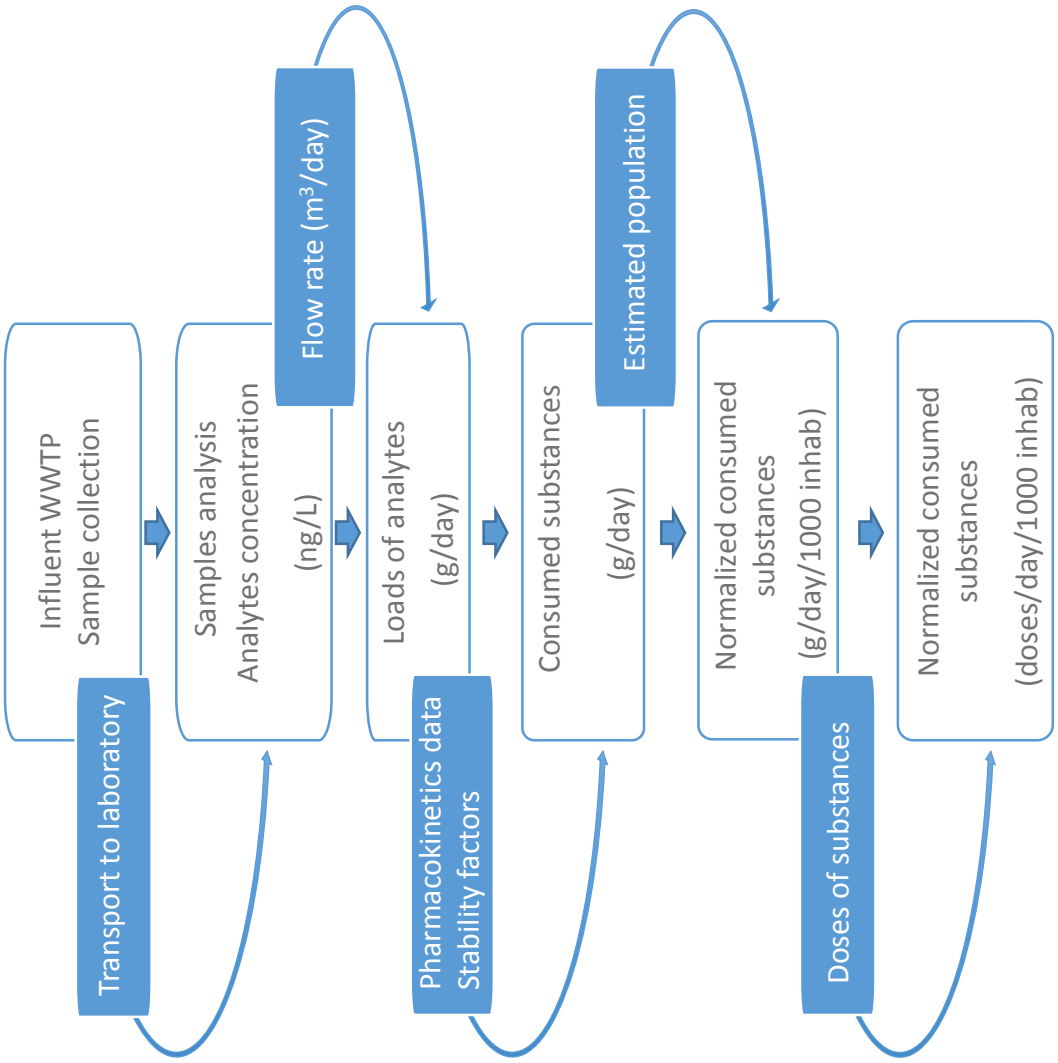

Fig 1.1. Scheme of the workflow in wastewater based epidemiology

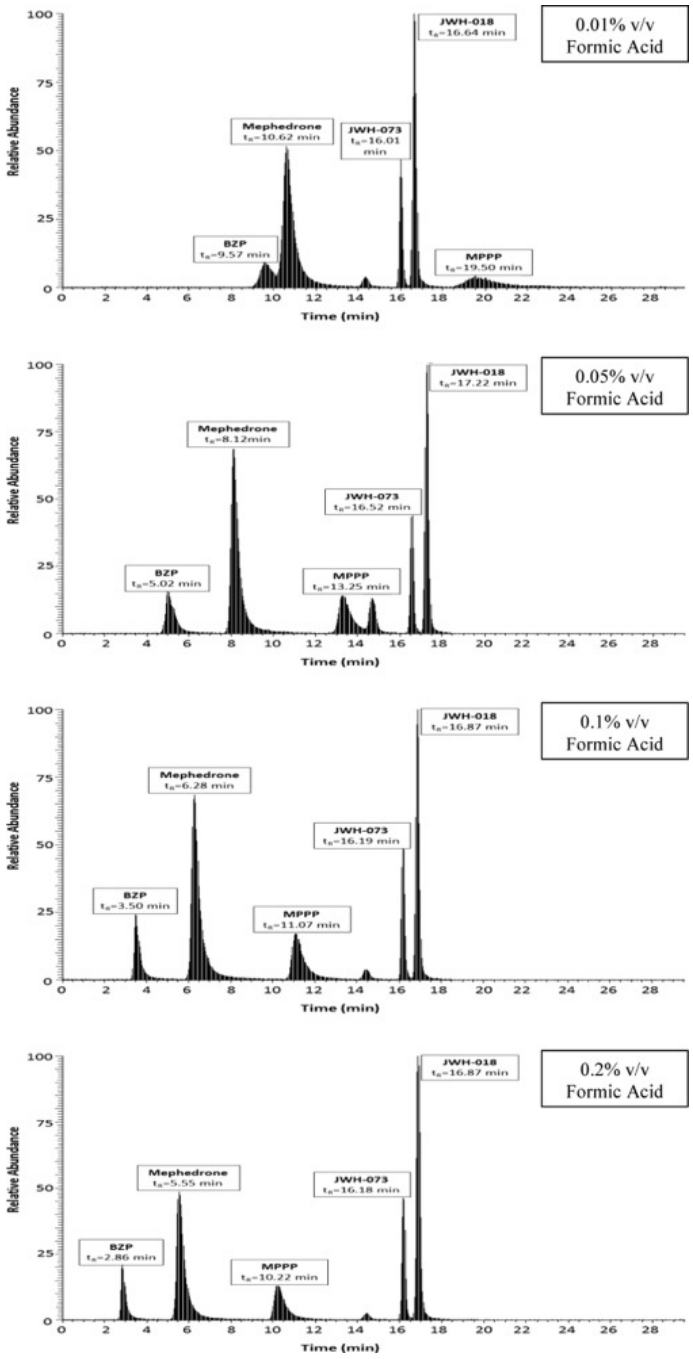

Fig 1.2. Comparison of the total Ion Chromatograms (TIC) of new psychoactive substances using three different stationary phases (concentration: 80 ng/L). Reproduced with permission from Ref. [25] Copyright (2015) Elsevier B.V.

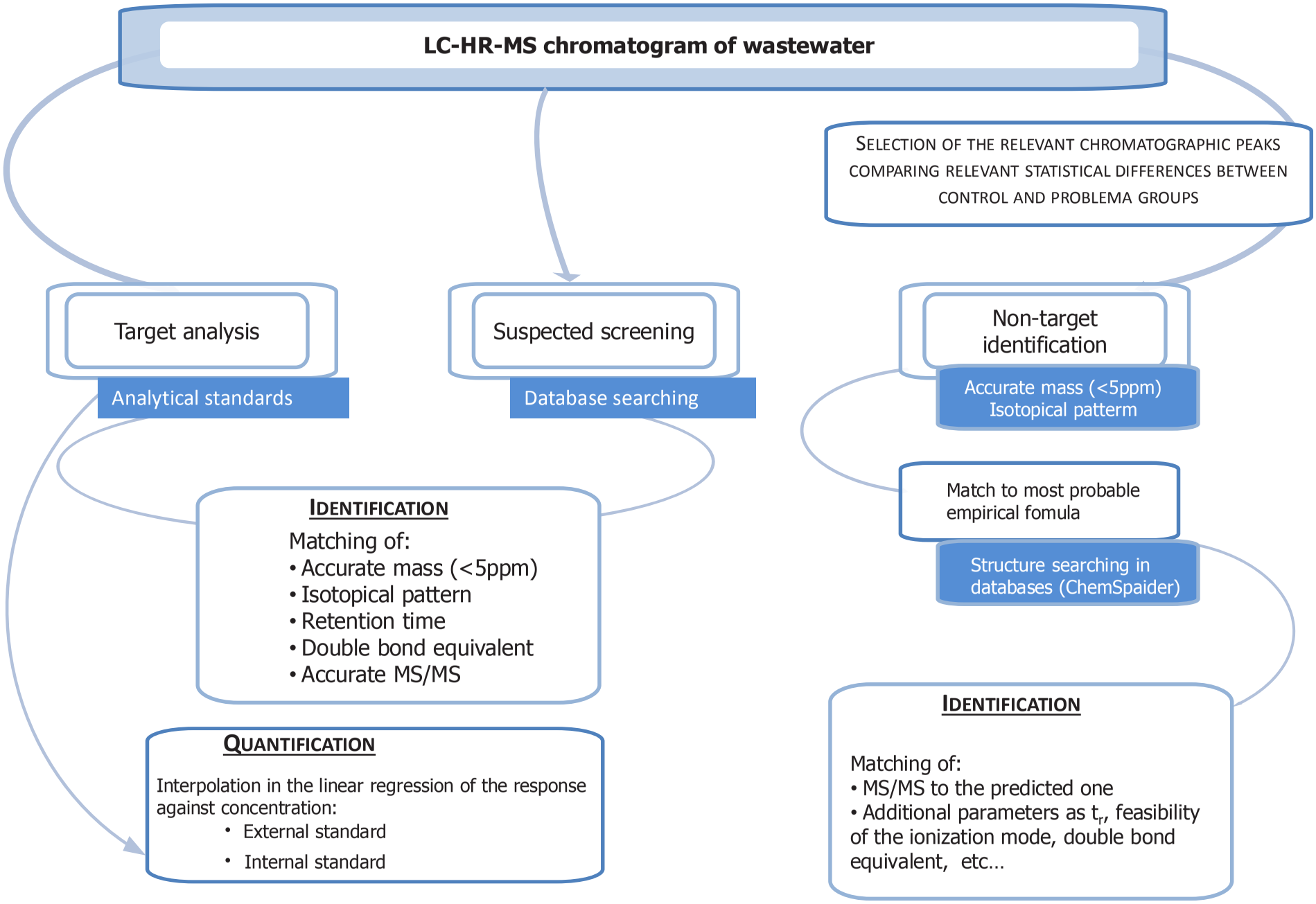

Fig 1.3. Different working modes with HR-MS

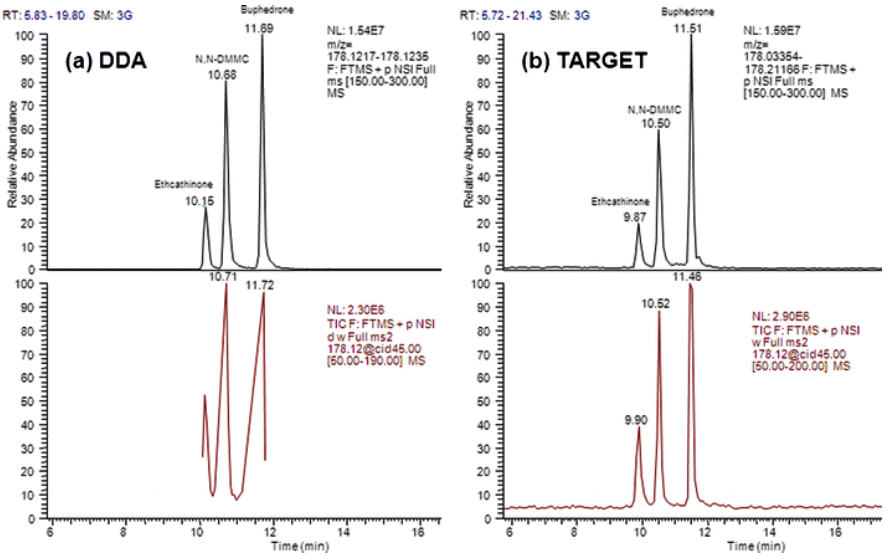

Fig 1.4. Extracted ion chromatogram (EIC) of the parent m/z 178.1226 (actually three different isomers: ethcathinone, N,N-dimethylcathinone, and buphedrone) and total ion chromatogram (TIC) of its MS/MS product ions, acquired in DDA mode (a) and target mode (b). Reproduced with permission from Ref. [26] Copyright (2016) Springer.

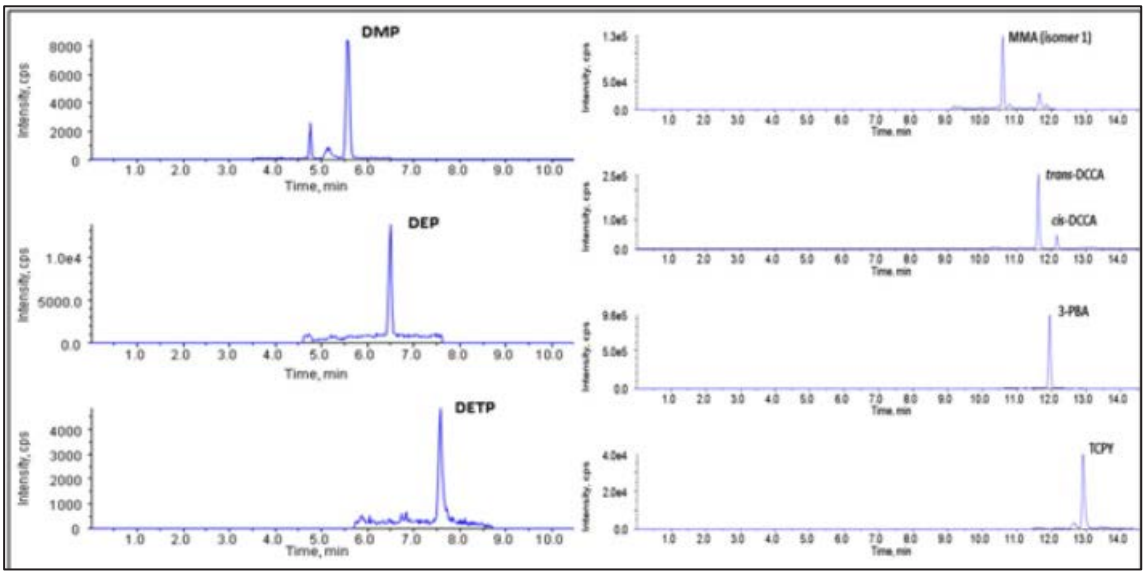

Fig 1.5. Characteristic chromatograms of some pesticide metabolites in influent wastewater (Concentrations (ng/L): DMP 483; DEP 206; DETP 70; MMA (isomer 1) 207; trans-DCCA 298; cis-DCCA 141; 3-PBA 181; TCPY 30 [57].

Peak ID	Name	Formula	m/z	ppm	R.T. (min)	Peak Area	% Area	% Score
M6	Loss of CH2 +Oxidation	C9H11NO2	166.0861	-0.9	1.91	2.38E+05	0.1	89.6

Fig 1.6. A) Identification of an EPH metabolite B) MS and C) MS/MS formed by demethylation and oxidation obtained using the software Metabolite Pilot 2.0. Reproduced with permission from Ref. [24]. Copyright (2016) Elsevier B.V

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Supplementary content

Liquid chromatography-mass spectrometry as a tool for wastewater-based epidemiology

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List of drugs of abuse abbreviations

5-HIAA: 5-hydroxyindoleacetic acid
6MAM: 6-acetylmorphine
ACOD: acetylcodeine
ADEP: antidepressants
AECME: anhydroecgonine methyl ester
ALP: alprazolam
AMP: amphetamine
BE: benzoylecgonine
BDB: benzodioxazolylbutanamine
BPPZ: benzyl piperazine
BUF: bufotenine
BUP: buprenorphine
BZD: benzodiazepines
CATH: cathinone
COC: cocaine
COCET: cocaethylene
COD: codeine
COT: cotinine
DCOD: dihydrocodeine
ECME: ecgonine methyl ester
ECG: ecgonidine
EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EMDP: 2-ethyl-5-methyl-3,3-diphenylpyrroline
EPH: ephedrine
FEN: fentanyl
FLU: flunitrazepam
HER: heroin
HMMA: 4-hydroxy-3-methoxymethamphetamine
KET: ketamine
LSD: lysergic acid diethylamine
MAMP: methamphetamine
MESC: mescaline
MBDB: methylbenzodioxolylbutanamine
MCATH: methcathinone
MDMA: 3,4-Methylenedioxy-N-methylamphetamine
MDEA: 3,4-Methylenedioxy-N-ethylamphetamine
MDA: 3,4-Methylenedioxyamphetamine
MDPV: methylenedioxypyrovalerone
MESC: mescaline
MET: methadone
METONE: methylone
MEP: mephedrone
MEPHEN: methylphenidate
MID: midazolam
MOR: morphine
MQUA: methaqualone
M3G: morphine-3-β-D-glucuronide
M6G: morphine-6-β-D-glucuronide
NBECG: norbenzoylecgonine
NBUP: norbuprenorphine glucuronide
NCOC: norcocaine
NCOD: norcodeine
NEPH: norephedrine
NFEN: norphentanyl
NMOR: nomorphine
NPOPH: norpropoxyphene
NTRA: nortramadol
OXY: oxycodone
OXA: oxazepam
OHLSD: 2-Oxo-3-Hydroxy-LSD
OMOR: oxymorphone
PCY: phenycyclidine
POPH: propoxyphene
PXT: paraxanthine
SDEN sildenafil
TFMPP: 1-(3-trifuoromethylphenyl) piperazine
THC: Δ-9-Tetrahydrocannabinol
THCA: 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid
THCCOOH: 11-Nor-9-carboxy-Δ-9-tetrahydrocannabinol
TRA: tramadol
ZOL: zolpidem

Other abbreviations:

AA: Acetic acid
ACN: Acetonitrile
AmA: Ammonium acetate
AmF: Ammonium formate
AmH: Ammonium hydroxide
BOD: biochemical oxygen demand
COD: chemical oxygen demand
FA: Formic acid
HESI: Heated electrospray ionization source
MeOH: methanol
N: nitrogen
P: phosphorous
TEA: Triethylamine

Table S1.1. Overview on studies related to the determination of drugs of abuse by LC-MS in WBE all over the world

Location	Drugs	Extraction	Recoveries (%)	LC separation		Mass Spectrometry	LODs (ng/L)	Estimated Population	Ref.
				Column	Mobile phase				
Korea	COC, BE, AMP, MAMP, MDMA, MDEA, MDA, COD, MOR, MET, EDDP, KET, NKET, MEP, METONE, THC	SPE. 200 mL filtered (pH 3) sample through Oasis MCX. analytes eluted in MeOH and 2% AmH in MeOH	94 – 125	Luna C18 150 × 2 mm, 3 µm	Gradient ACN – water both 0.1% FA at 0.35 mL/min	QqQ ABSciex, QTRAP®5500 SRM ESI (+)	0.03 – 7.5	Provided by the WWTP personnel	(Kim *et al.*, 2015)
	THC-COOH				Gradient MeOH – water both 0.1 % AA at 0.35 mL/min	ESI (-)			
USA	AMP, MAMP,MDA, MDEA, MDMA; THC, THC-COOH, COC, BECG, COCET, MOR, 6MAM, COD, OXY, HER, MET, EDDP, LSD	ON LINE SPE. Thermo EQuan MAX system with HyperSep™ Retain PEP. Analytes eluted in MeOH	83 – 163	Hypersil C18 Gold™ aQ (150 × 2.1 mm, 3 µm) protected by a Hypersil Gold™ aQ guard column (10 × 2.1 mm, 3 µm)	Gradient water – MeOH – 1% FA/100 mM AmF buffer at 0.5 mL/min	Q Exactive™ hybrid Quadrupole-Orbitrap Scan range from 100 to 500 m/z HESI-II (+)	0.6 – 1.7	Minimum and maximum consumption levels using both the total campus population and the maximum number of daily occupied parking spaces	(Heuett *et al.*, 2015)
Australia	COC, BECG, AMP, MAMP, MDMA	SPE. 200 mL acidified and filtered sample through Oasis MCX. Analytes eluted in MeOH and 2% AmH/MeOH	75 – 81	Luna C18 (150 × 3mm, 3 µm)	Gradient ACN – water both 0.1 % FA at 0.3 mL/min	QqQ AB Sciex Qtrap® 5500 MRM ESI (+)	< 1 µg/L	Based on census data and on determined biomarkers (atenolol, COD,	(Lai *et al.*, 2015)

								caffeine, hydrochlorthiazide, acesulfame, salicylic acid, carbamazepine, naproxen)	
Canada	COC, BE, AMP, MDA, MAMP, MDMA, EPH	SPE. 100 mL filtered (pH 2.5) sample through Oasis MCX. Analytes eluted in 5% AmH/MeOH	83 – 101	Genesis C18 (150 × 2.1 mm 4 µm) protected by a guard column C18 (4 mm × 2.0 mm)	Gradient water with 0.1% FA – ACN at 0.32 mL/min	QqQ API 3000 MRM ESI (+)	0.01 – 5.1	Not specified	(Yargeau *et al.*, 2014)
	COD, ACOD, DCOD, MOR, 6MAM,MET, HER, TRA, KET, OXY, EDDP				Gradient water with 10 mM AmA and 0.1% AF – ACN at 0.24 mL/min				
China	COC, BECG, ECME, 6MAM, MET, EDDP, AMP, MDMA, MAMP	SPE. 50 mL filtered sample through Oasis MCX. Analytes eluted in MeOH and 5 % NH_3/MeOH		Luna HILIC column (150 mm × 3 mm, 5 µm)	Gradient water with 5 mM AmA – ACN at 0.4 mL/min	QqQ Agilent 6410 MRM ESI (+)	1 – 20*	Population provided by the WWTP personnel based on census data.	(Khan et al., 2014)
	MEP, MDPV, KET, NKET	SPE. 50 mL filtered sample through Oasis MCX. Analytes eluted in MeOH	51 – 99	Gemini C18 (50 mm × 2 mm, 3 µm) protected by a C18 guard column (4 mm × 2 mm)	Gradient water with 5 mM AmA – ACN at 0.35 mL/min				
	THC-COOH					ESI (-)			
Finland	METONE, AMP, MDA, MAMP, MDMA BECG, COC, MDPV, EDDP	SPE. 40 mL (pH 2.5) centrifuged sample trough Oasis MCX. Analytes eluted in 3% NH_3/MeOH	93 – 100	Acquity CSH C18 (2.1 mm × 75 mm, 1.7 µm) and protected by a guard column	Gradient water, 5 mM AmF and 0.05% FA (pH 3.4) – ACN at 0.5 mL/min	QqQ 6460 dynamic MRM ESI (+)	Not specified	Population provided by the WWTP personnel	(Kankaanpää et al., 2014)

				Acquity CS C18 VanGuard (2.1 mm × 5 mm, 1.7 µm)					
France	COC, BECG, NCOC ECME, COCET, MOR, 6MAM, HER, MDMA, MDA, MDEA, MAMP, AMP, MET, EDDP BUP,	SPE. 200 mL filtered sample through Oasis HLB. Analytes eluted in MeOH	61 – 124	Xbridge Phenyl (3mm × 150mm, 3.5 mm)	Gradient water and 5 mM AmF (pH 4) – ACN at 0.4 mL/min	QqQ Quantum Access Max SMR ESI (+)	5 – 40	Based BOD	(Damien et al., 2014)
	THC-COOH					ESI (-)			
USA	COC, BECG, NCOC, COCET, MOR, M3G, M6G, MET, EDDP, AMP, MAMP, MDA, MDMA, MDEA, PXT	SPE. 100 mL centrifuged and filtered sample through Oasis HLB. Analytes eluted in MeOH and acetone/MeOH/ethyl acetate (2:2:1) and 5% ammonia/MeOH	101 ± 27	Ultra Biphenyl (100 mm × 2.1 mm, 5 µm)	Gradient water with 0.1% FA – MeOH at 0.4 mL/min	QqQ API 2000 MRM ESI (+)	0.1 – 5	Not specified	(Subedi et al., 2014)
Switzerland	MDMA, COC, BE, THCCOOH MDMA, HMMA	SPE. 500 mL filtered (pH 2) sample through Oasis MCX. Analytes eluted in MeOH and MeOH/AmH	49 – 74	Capillary column HP-5 MS (20 m × 0.18 mm, 0.18 µm)	At 0.9 mL/min	QqQ Agilent 7000B MRM	1 – 5*	Based on census, ammonium (ion selective electrode), P, and COD	(Been et al., 2014)
Portugal	COC, BECG, COT	SPE. 100 mL (pH 6) centrifuged and filtered sample	92 – 109	Luna HILIC 5 µm 200A (150 × 3 mm)	Gradient water with AmA – ACN at 0.3 mL/min	Single quadrupole MicroMass	1 – 15	Based on P, N, BOD and COD	(Lopes et al., 2014)

		through Oasis HLB. Analytes eluted in MeOH				Quattro micro® API SRM ESI(+)			
Slovakia	AMP, BECG, COC, MDA, MDEA, MDMA, MAMP, MEPHEN, THC-COOH	ON LINE SPE. 10 mL filtered through C18 Hypersil Gold. Analytes eluted in water and MeOH both with 0.1 % FA	57 – 125	Cogent bidentate o C18 (50 mm × 2.1 mm i.d., 3 µm particles preceded by a guard column (10 mm × 2.1 mm i.d, 3 µm particles)	Gradient water – ACN both with 0.1% FA at 0.3 – 0.35 – 0.4 mL/min	Q-Exactive, Hybrid quadrupole Orbitrap high resolution mass spectrometry (HRMS) HESI-II (+)	2 – 9.4*	Theorical equivalents for connected people and real connected population	(Mackuľak et al., 2014)
Sweden	AMP, BECG, CATH, 6MAM, COC, HER, KET, MBDB, MEP, MDEA, MAMP, MEPHEN, MID, NKET, LSD, OHLSD, OXY EDDP, MET, BUP, NBUP, ALP, COD, FEN, FLU, MOR, OXA, ZOL, TRA	ON LINE SPE. 1 mL filtered (pH 3) sample through Oasis HLB (2.1 x 20, 15 µm). Analytes eluted in MeOH	60 – 135	Hypersil GoldAQ C18, 50 × 2.1 mm, 5 µm preceded by a guard column	Gradient water – MeOH both with 0.1% FA at 0.2 - 0.25 - 0.35 mL/min	QqQ TSQ Quantum Ultra SRM ESI (+)	1 – 50*	Population provided by the WWTP personnel	(Östman et al., 2014)
Spain	6MAM, AMP, BECG, COC, KET, MAMP, MDMA, THC-COOH	SPE. 250 mL filtered through Strata X 33 polymeric reversed phase. Analytes eluted in MeOH	66 – 114	Kinetex C18 (1.7 mm, 100 A, 50 × 2.10 mm)	Gradient water – MeOH both with 0.1% FA at 0.2 mL/min	QqQ Agilent 6410 MRM ESI (+)	1	Based on COD	(Andrés-Costa et al., 2014)

Spain	BUF, COCET, MOR, THC-COOH	SPE. 250 mL filtered sample through Strata X 33 polymeric reversed phase. Analytes eluted in MeOH	61 – 101	Kinetex C18 (1.7 mm, 100 A, 50 × 2.10 mm)	Gradient water – MeOH both with 0.1% FA at 0.2 mL/min	QqQ Agilent 6410 MRM ESI (+)	4 - 14	Based on BOD, COD, P and N and selected biomarkers (caffeine, cotinine and 5-HIAA)	(Rico *et al.*, 2017)
Poland	AMP, MAMP, MDA, MDMA, MDEA	SPE. 10 L filtered (pH 7) sample through Bakerbond Narc-2 mixed mode. Analytes eluted in water and hydrochloric acid/MeOH	80 – 93	Zorbax XDB C18, (4.6 × 50 mm × 1.8 µm)	Gradient water with formic buffer (pH 3.2) – ACN at 0.45 mL/min	QqQ Agilent 6410B MRM ESI (+)	0.71 – 1.77	Not specified	(Nowicki et al., 2014a)
Australia	MDMA, AMP, BECG, COC, MAMP, BPPZ, MEP, METONE	SPE. 200 mL filtered sample through Oasis MCX. Analytes eluted in MeOH.	89 – 105	Luna C18 (150 × 2 mm, 3 µm)	Gradient water – ACN both with 0.1% FA at 0.35 mL/min	QqQ AB SCIEX QTRAP®5500 MRM ESI (+)	0.03 – 3.02	Based on ticket sales, the number of daily attendees	(Lai, Thai, et al., 2013)
	THC-COOH				Gradient water – MeOH both with 0.1% AA at 0.4 mL/min	ESI (-)			
France	COC, BECG, NCOC, ECME, COCET, 6MAM, MOR, HER, MDMA, MDA, MDEA, MAMP, AMP, MET, EDDP, BUP	SPE. 250 mL filtered sample through Oasis HLB. Analytes eluted in MeOH	61 – 124	Xbridge Phenyl (3.5 mm, 3 mm × 150 mm)	Gradient water with 5 mM AmF (pH 4) – ACN at 0.4 mL/min	QqQ Quantum Access Max SMR ESI (+)	5 - 40	Population provided by the WWTP personnel	(Nefau et al., 2013)

	THC-COOH					ESI (-)			
Finland	THCA, AMP, MAMP, MDMA, 6MAM, EDDP, BECG, MOR MDPV, MET	100 mL centrifuged sample through Oasis HLB. Analytes eluted in MeOH	82 – 144	Gemini-NX C18 (100 mm × 2.0 mm, particle size 3 µm) protected by a guard column Kinetex C18 (4.0 mm × 2.0 mm)	Gradient water with 10 mM AmA buffer (pH 3.2) – MeOH with 0.1% FA at 0.3 mL/min	QqQ AB Sciex 4000 QTRAP® SRM ESI (+)	0.1 – 100	Based on P, N, BOD and COD	(Vuori et al., 2014)
England	COC, BECG, NBECG, NCOC, COCET, AECME, ECG, AMP, MAMP, MCATH, BPPZ TFMPP,MDA, MDMA, MDEA MBDB, BDB, MESC, LSD, OHLSD,HER, 6MAM, COD, NCOD, OXY, OMOR, MOR, NMOR, DCOD, BUP, NBUP, MET, EDDP, EMDP, FEN, NFEN, POPH, NPOPH, TRA, NTRA, KET, PCY,	SPE. 100 mL filtered (pH 1.8 – 1.9) sample through Oasis MCX. Analytes eluted in 7% AmH/MeOH	39 – 226	AQUITY UPLC BEH C18 (1.7 µm; 1mm × 150 mm)	Gradient water – MeOH – AA (pH 2.9) at 0.04 mL/min	QqQ Waters SRM ESI (+)	0.1 – 26.5	Not specified	(Baker et al., 2014)

	BZD, ADEP, MQUA, SDEN, EPH, NEPH								
Poland	AMP, MAMP, MDA, MDMA, MDEA	SPE.10 L filtered (pH 7) sample through Bakerbond Narc-2 mixed mode. Analytes eluted in water and hydrochloric acid/ MeOH	80 – 93	Zorbax XDB C18 (4.6 × 5 0mm, 1.8µm)	Gradient water formic buffer (pH3.2) – ACN at 0.45 mL/min	QqQ 6410B MRM ESI (+)	0.71 – 1.77	Not specified	(Nowicki et al., 2014b)
Hong Kong	COC, BECG, AMP, MAMP, MDMA, KET, NKET	200 mL filtered (pH2) sample through Oasis MCX. Analytes eluted in MeOH and 2% AmH/MeOH	89 – 104	Luna C18 (150 × 2 mm, 3 µm)	Gradient water with 0.1% FA – ACN at 0.35 mL/min	QqQ AB SCIEX QTRAP®5500 MRM ESI (+)	0.10 – 2.25	As such, we had to rely on a nominal figure for the population contributing to the wastewater treatment plant and assumed that this population was consistent throughout the study period	(Lai, Bruno, et al., 2013)
England	AMP, MAMP, EPH, MDA, MDMA	100 mL filtered (pH 7.5) sample through Gilson ASPEC XL4 and Oasis HLB. Analytes eluted in MeOH	78 – 97	Chiral-CBH, (100 × 2 mm, 5 µm) protected by a guard column Chiral-CBH (10× 2.0 mm)	Water – 2-propanol with 1 mM AmA (pH 5.0) at 0.075 mL/min	QqQ waters MRM ESI (+)	0.6 – 5.6*	Not specified	(Kasprzyk-Hordern et al., 2012)
USA-Pacific Northwest	Caffeine MAMP BECG	7 mL centrifugued sample. Large volume injection	--	Atlantis T3 C18 (150 × 4.6 mm × 5 µm)	Graient water with 0.1% acetic acid in MeOH -	QqQ Waters Quattro Micro MRM	5 – 2000*	The measured creatinine loads were used to	(Brewer et al., 2012)

	COC			protected by a security guard cartridge C18 (2.0 × 4.0 mm)	ACN at 0.5 mL/min	ESI (+)		estimate the population utilizing the WWTP during the study period	
	Creatinine			Luna C18 (150 × 4.6 mm 5 µm) protected by a guard column C18 (2.0 × 4.0 mm)	Gradient water with 10 mM AmA – MeOH. Isocratic conditions at 0.5 mL/min		50000*		
Brussels	BECG, ECME, AMP, MAMP, MDMA, MET, EDDP, 6-MAM	50 mL filtered sample through Oasis MCX. Analytes eluted in MeOH and 5% NH_3/ MeOH.	> 60 % except for ECME (35 %)	Luna HILIC (150mm × 3 mm, 5 µm)	Gradient water with 5 mM AmA – ACN at 0.4 mL/min	QqQ Agilent 6410 MRM ESI (+)	1 – 2*	Based on P, N, BOD and COD	(van Nuijs et al., 2011)
USA Oregon	MAMP	7 mL centrifugued sample. Large volume injection		Atlantis T3 C18 (150 × 4.6 mm × 5 µm) protected by a guard column C18	Gradient water with 0.1% AA in MeOH – ACN at 0.5 mL/min	QqQ Waters Quattro Micro MRM ESI (+)	10*	Not specified	(Chiaia-Hernandez et al., 2011)
Italy	BECG, MAMP, MOR, 6MAM	50 mL filtered (pH 2) sample through Oasis MCX. Analytes eluted in MeOH and 2% ammonia solution/MeOH.	51 – 112	XTerra C18 (100 × 2.1 mm, 3.5 µm)	Gradient water with 0.05% AA – ACN at 0.2 mL/min	QqQ API 3000 MRM EIS (+)	1.75 - 5.3*	Based on BOD	(Zuccato et al., 2011)
	THC-COOH				Gradient water with 0.05% TEA – ACN 0.2 mL/min	EIS (-)			
Australia	COC, BECG, ECME, AMP, MAMP, MDMA, MDEA, MDA, THC , MET, EDDP, COD, MOR	200 mL filtered (pH2) sample through Oasis MCX. Analytes eluted in MeOH and 2% AmH/ MeOH	83 – 113	Luna C18 (150 × 2 mm, 3 µm)	Gradient water with 0.1% FA – ACN at 0.35 mL/min	QqQ AB SCIEX QTRAP®5500 MRM EIS (+)	0.03 – 3.02	Based on census data and human-specific biomarker atenolol	(Lai et al., 2011)
	THC-COOH					EIS (-)			

* LOQ

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Capítulo 2

CAPÍTULO 2

METODOLOGÍAS ANALÍTICAS

En el Capítulo 2 se detallan las metodologías analíticas desarrolladas y validadas para determinar las sustancias seleccionadas en diferentes matrices ambientales (aguas, lodos activos y sólidos en suspensión procedentes de WWTPs, y aguas superficiales y sedimentos procedentes de la cuenca del río Turia). Las metodologías desarrolladas basadas en el análisis LC-MS/MS y UHPLC-QqTOF-MS/MS se utilizaron para analizar los compuestos seleccionados. Los resultados obtenidos dentro de este capítulo se estructuran en tres publicaciones focalizadas en el desarrollo de métodos de identificación y análisis de estos compuestos.

- Publicación científica 2. *Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter*
- Publicación científica 3. *Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer*
- Publicación científica 4. *Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight*

PUBLICACIÓN CIENTÍFICA 2

Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter

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Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter☆

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ABSTRACT

An analytical method for determining traditional and emerging drugs of abuse in particulate matter, sewage sludge and sediment has been developed and validated. A total of 41 drugs of abuse and metabolites including cocainics, tryptamines, amphetamines, arylcyclohexylamines, cathinones, morphine derivatives, pyrrolidifenones derivatives, entactogens, piperazines and other psychostimulants were selected. Samples were ultrasound extracted with McIlvaine buffer and methanol, and the extracts were cleaned up by solid phase extraction (SPE) using Strata-X cartridges. Drugs were eluted using methanol and methanol–dichloromethane and determined by liquid chromatography tandem mass spectrometry. The optimum solid–liquid extraction (SLE) conditions were: weight 1 g of sample and ultrasound assisted extraction (UAE) with 10 mL of methanol–McIlvain buffer (1:1, v/v, pH 4.5) for 10 min. Recoveries for all compounds were ≥50% in the three matrices with the exception of ephedrine (EPHE), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), ecgonine methyester (ECME), heroin (HER), 3,4-methylendioxyamphetamine (MDA) and 4-acetoxy N,N′-dimethyltryptamine (4-AcO-DIPT) and methadone (MET). Data acquisition was done by selective reaction monitoring (SRM), and the two most abundant product ions were used for confirmation. Limits of detection were lower than 1.32 ng g^{-1} dry weight (d.w.) and limits of quantification were between 0.12 and 3.96 ng g^{-1} (d.w.). The method was applied to the analysis of particulate matter, where cocaine (COC), benzoylecgonine (BECG), ecgoninemethylester (ECME), cocaethylene (COCET), methadone (MET) and codeine (COD) were mostly detected. In the case of dehydrated sludge, opioids are at higher concentration than cocainics and some emerging drugs such as 4-methoxyamphetamine (PMA), ketamine (KET) and bufotenine (BUF) were detected. In sediment COC, 4-methoxyphencyclidine (4-MeO-PCP), MET and BECG were most relevant compounds.

1. Introduction

Drugs are chemical agents that alter the biochemical or physiological processes of tissues or organisms [1], particularly, drugs of abuse are those whose use does not pursue a medical purpose. At present, they are separated into "emerging" and "traditional" drugs of abuse. The former includes those that were not listed in the 1961 United Nations Single Convention on Narcotic Drugs and in the 1971 United Nations Convention on Psychotropic Substances [2–4]. Although some of them, as tobacco and alcohol, are legal in Spain and other countries, most of these drugs are illegal. These drugs are metabolized by the body and both, unchanged compounds and their metabolites, are primarily excreted in the urine [5–9].

Some studies of drugs at the influents and effluents of wastewater treatment plants (WWTP) demonstrate a variable elimination rate (between 45 and 95%) depending on the drug [10]. For compounds such as cocaine (COC) and amphetamine (AMP) elimination efficiency is over 90%, while for other drugs or metabolites such as ecstasy, methamphetamine (MAMP), 11-nor-9-carboxy tetrahydrocannabinol or LSD is much less [11]. Further research is needed to determine the illicit drugs released to the environment and their possible impact on it. Part of these drugs could become deposited in fluvial sediments, which are not renewed as quickly as the water causing a possible long-term accumulation depending on their stability. The particulate matter present in wastewater influent has scarcely been studied [12,13]. These studies show that a significant

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fraction of illicit drugs ends in the particulate matter. The sludges generated by WWTP are often used as agricultural fertilizer, in some instances, after composting processes. Numerous studies have confirmed the presence of contaminants, including illicit drugs in sludge [14–17] that could pose a risk to agricultural soils and plants. The composition and quantity of sludge generated depends on the treatments applied in the WWTP and the wastewater composition. Even within the same WWTP, their characteristics may change annually, seasonally or daily due to variations in the composition of the influent wastewater and daily variations in treatment processes [18]. Monitoring of particulate matter and sludge from the WWTP and sediments in the receiving waters is crucial.

Table 2.1 outlines the methods published up to now, to extract and quantify illicit drugs in solid matrices including soil, sediment, sewage sludge and particulate matter. The study of illicit drugs in these matrices is very recent, the oldest method back to 2006 but the next was published in 2010. It is necessary to develop methods that provide a large percentage of recovery and high sensitivity. Most common extraction techniques are pressurized liquid extraction (PLE) [13,14,16,17,19] or solid–liquid extraction (SLE) [12,15,20] followed by a solid phase extraction (SPE) clean-up [12,13,15,17,19,20]. PLE is the preferred approach because this technique has as advantage that high pressure solvents remain in the liquid state above their boiling points. Therefore, these conditions enhance the solubility of target compounds in the solvent and the desorption kinetics from solid matrices providing shorter extraction times and great reproducibility [13,16,19,21]. However, this technique (i) requires special instrumentation to reach simultaneously high pressure and temperature, (ii) is expensive (several g of sorbents and mL of solvents, N_2 stream, and energy consumption) and (iii) matrix compounds are also frequently co-extracted. Furthermore, a number of studies reports similar recoveries using more conventional SLE by shaking or sonication for moderate and polar analytes, as illicit drugs [12,14,21].

The aim of this research was to develop and optimize analytical methods to determine 41 illicit drugs in sediments, sludges and particulate matter (see Table S2.1 in the supplementary material for the detailed list of compounds and their physico-chemical properties [22]). These compounds include traditionally consumed drugs of abuse (some of them as morphine (MOR) and codeine (COD) nowadays have mostly a medical use) and emerging psychoactive drugs. The method is based on traditional SLE favored by ultrasonication, followed by SPE clean-up and liquid chromatography tandem mass spectrometry (LC–MS/MS) determination. To the best of our knowledge there is not extraction method reported for determining ethylamphetamine (ETAMINE), ethylone (ETONE), methylphenidate (MEPHEN), mephedrone (MEP), methylone (METONE), dibuthylone (bk-MMBDB), 4-bromo-2,5-dimethoxyphenethylamine (2C-B), naphyrone (NAPH), methylenedioxypyrovalerone (MDPV), p-methoxyamphetamine (PMA), 4-acetoxy N,N′-diisopropyltryptamine (4-AcO-DIPT), bufotenine (BUF), 1-(3-chlorophenil)piperazine (mCPP), α-pyrrolidinopropiophenone (PPP), α-pyrrolidinopentiophenone (α-PVP), 3,4-methylendioxy-α-pyrrolidinopropiophenone (MDPPP), 4-methyl-α-pyrrolidinopropiophenone (4-MePPP), 4′-methyl-α-pyrrolidinohexanophenone (4′-MePHP), 4′-methyl-α-pyrrolidinobutiophenone (MPBP), 3-methoxyphencyclidine (3-MeO-PCP) and 4-methoxyphencyclidine (4-MeO-PCP) in sediments, sludges and particulate matter. The method was applied to determine these substances in sewage sludge and particulate matter from the WWTP Pinedo I and II, and Quart-Benàger, and in sediments taken from the Turia river (Valencia, Spain). The incidence of many of these compounds in sediment and particulate matter is assessed for the first time providing information on their presence in environmental matrices.

2. Experimental

2.1. Reagents and materials

The methanol used was LC–MS PAI 99.9% purity distributed by Panreac (Barcelona, Spain). The dichloromethane was 99.8% pure, stabilized with 0.1% ethanol and distributed by VWR® BDH Prolabo® (Barcelona, Spain). Formic acid was from AMRESCO® (Solon, OH, USA), citric acid from PROBUS S.A. (Badalona, Spain), and Na_2HPO_4 from Panreac. All of them were analytical grade.

AMP, MAMP, Ephedrone (EPHED), ETONE, MEPHEN, MEP, METONE, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylendioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), N-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), bk-MMBDB, NAPH, PMA, 4-AcO-DIPT, BUF, 1-(3-trifluoromethylphenyl)piperazine (TFMPP), PPP, α-PVP, MDPPP, 4-MePPP, 4′-MePHP, MPBP, 3-MeO-PCP, 4-MeO-PCP, ketamine (KET), COC, benzoylecgonine (BECG), cocaethylene (COCET), ecgoninemethylester (ECME), COD, heroin (HER), methadone (MET) and MOR were distributed by LoGiCal® Standards (Barcelona, Spain). 6-monoacetylmorphine (6-MAM), ETAMINE, ephedrine (EPH), 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 2C-B and mCPP were distributed by Cerilliant® (Round Rock, TX, USA). MDPV was distributed by Toronto Research Chemicals Inc. (Toronto, Canada). BECG-d3, COC-d3, COCET-d3, EDDP-d3, MET-d3, MAMP-d5, MDMA-d5, MDEA-d5, METONE-d3 were distributed by LoGiCal® Standards. AMP-d5, MDA-d5, KET-d4, ECME-d3, 6-MAM-d3, HER-d9 and MOR-d3 were from Cerilliant®. Analytical standards and isotopically labeled internal standards were stored at −20 °C in dark (Table S2.1 of the supplementary material).

Water samples were filtered through GA-55 filters 90 mm and 0.45 μm pore diameter from ADVANTEC (Toyo Roshi Kaisha, Ltd. Tokyo, Japan) and deionized water through hydrophilic membrane propylene filters, with a diameter of 47 mm and a pore size of 0.2 μm manufactured by PALL Corporation (Mexico DF, Mexico). McIlvaine buffer pH 4.5 was prepared mixing 90.85 mL of 0.062 M Na_2HPO_4 solution and 9.5 mL of Citric acid 0.091 M and dilution to 1 L with distilled water. The lyophilizer used was a 4KBTXL-75 by VirTis SP Scientific of industries (Philadelphia, PA, USA). The equipment used for SPE was a VISIPREP™ from Supelco (Madrid, Spain). SPE was carried out on Strata-X Polymeric Reversed phase cartridges 200 mg/6 mL Phenomenex (Torrance, CA, USA). Samples were evaporated using a combined sample concentrator and a heating plate, the concentrator model was SBHCONC/1 and the heating plate model SBH130D/3 both manufactured by Stuart® (Stafford, UK). The vials used to inject the sample in the chromatograph were 2 mL amber vials with stoppers 99 mm + Septum Sil/PTFE, both manufactured by Análisis Vínicos S.L. (Tomelloso, Spain). Finally, syringe filters were Teflon (PTFE) hydrophobic with a pore size of 0.22 μm and manufactured by MS® (Ontario, Canada).

2.2. Sampling

Sludge and particulate matter samples were collected from three WWTPs that treat the sewage waters of Valencia and its orbital cities with a project flow rate of 60,000 m^3 day^{-1} (Quart-Benàger) [23] and 325,000 m^3 day^{-1} (the complex Pinedo I and II) [24]. Samples of WWTP were taken daily for seventeen consecutive days from 4th March to 20th of 2014. Influent wastewater samples (250 mL) were filtered under vacuum using the ADVANTEC® filters to retain the particulate matter. Then, filters were dried at room temperature for 24 h, weighted to compare the result with the mass of unused filters and determine the particulate matter weight (ranging from 2 to 5 g) and then frozen at −20 °C until the particulates were extracted.

Table 2.1
Summary of different extraction procedures published to determine illicit drugs in solid environmental matrices.

Family of compounds	Sample	Extraction	Sample amount	Final volume (mL)	Determination	Extraction time (min)	N° of compounds	LOD	Recoveries (%)	Reference
Illicit drugs (Amphetamine)	Sewage sludges (1 g)	SLE (50 mM) formic acid and methanol (80:20v/v). Adjusted at pH 10 SPE with Oasis HLB	1 g	1	LC–MS	–	1	2 $\mu g\,kg^{-1}$	90	[15]
Illicit drugs (Amphetamines and cocainics)	Particulate matter (collected from 11 L sewage water)	SLE (50 mM) formic acid and methanol (80:20v/v). Adjusted at pH 10 SPE with Oasis HLB	100 mL, 200 mL or filter	0.4	LC–MS/MS	21–31	6	1.3–7.22 $ng\,L^{-1}$ 0.01–0.16 $ng\,g^{-1}$	–	[12]
Pharmaceuticals and illicit drugs (Stimulants, hallucinogens, opioids, benzodiazepines, antidepressants, anesthetics)	Particulate material of sewage water and soils	PLE at 80 °C, 1500 psi and 60% flush for 5 min in 4 cycles. Adjusted to pH 2 and SPE with Oasis MCX	0.31 mg–1 g	0.5	LC–MS/MS	17–136	60	0.01–1.31 $ng\,g^{-1}$	4–145	[13,19]
Illicit drugs and metabolites (Cocainics, amphetamines, opioids, benzodiazepines, LSD, cannabinoids)	Sewage sludges	PLE Al_2O_3 at 50 °C, 1250 psi and 60% flush for 5 min in 1 cycle	0.5 g	1.5	LC–ESI-MS/MS	15	20	0.5–6.4 $ng\,L^{-1}$	5–135	[16]
Illicit drugs (Amphetamines, cannabinoids, and morphine derivatives)	Sewage water and sludges and particulate material	PLE (H_2O/MeOH (50:50v/v) and 50 mM H_3PO_4/MeOH (50:50v/v)) al 80 °C, 1500 psi and 120% flush in 5 cycles and SPE with Oasis MCX	125 mL or 250 mL	0.5	LC–MS/MS	15	13	0.1–3.1 $ng\,g^{-1}$	3–101	[17]
Illicit drugs and metabolites (Nicotine, opioids, alkaloids)	Sewage sludges	Mix with diatomaceous earth. PLE Cl_2CH_2 at 100 °C, 1500 psi and 60% flush for 7 min in 1 cycle	1 g	5	LC–MS/MS	22	9	0.5–10 $\mu g\,kg^{-1}$	44–95	[14]
Pharmaceuticals and illicit drugs	Sewage sludge	2 mL MeOH–Milli-Q water (pH 2.5, FA 0.5% and 0.1% EDTA), 50:50 (v/v)	0.1 g	0.5	LC–MS/MS	45	148	0.6–19.9 $ng\,g^{-1}$	17–126	[20]

Dehydrated sludge samples were also taken daily with the exception of Quart-Benàger because this WWTP does not prepare dehydrated sludge every day. In Pinedo I and Pinedo II, the sludge of both WWTPs were deposited in the same tank, so the analyzed samples were mix of both. Sediments sampling campaign was performed during 2013 along the Turia river (East of Spain). Sampling locations were up and down of the WWTPs. Once in the laboratory, sediments and dehydrated sludges were homogenized, frozen and lyophilized. The lyophilized samples were passed through a series of sieves with 2 mm, 500 μm and 125 μm of diameter to obtain a homogeneous mixture. Both, sludges and sediments, were stored in dark at −20 °C until extraction.

2.3. Extraction procedure

In the optimized procedure, one halve of the filters with particulate matter (ranging from 1 to 2.5 g) were cut in three pieces of similar size, or 1 g of sediment or sludge were placed in a 15 mL falcon tubes and were spiked with a mixture of the internal standard (IS) to obtain a final concentration of 25 $\mu g\,L^{-1}$ in the extract. Then, the extraction was performed with 10 mL of McIlvain buffer–methanol (1:1, v/v) using ultrasound assisted extraction (UAE). The samples were shaken 15 s to homogenize the mixture, sonicated for 10 min and centrifuged at 1200 rcf for 15 min. The supernatant was placed into a 250 mL volumetric flask, diluted with distilled water, adjusted to pH 6 with 1 M NaOH and bought to final volume with distilled water.

SPE cartridges were previously activated by passing 6 mL of methanol and 6 mL of deionized water. Then, the sample of 250 mL was passed through the cartridge (10 $mL\,min^{-1}$) under vacuum. The cartridges were washed with 5 mL of distilled water and dried under vacuum for 15 min. Analytes were eluted passing 6 mL of methanol and then 3 mL of a methanol–dichloromethane solution (1:1, v/v) at gravity flow. The extracts were evaporated to dryness under a gentle stream of N_2 and reconstituted with 1 mL of a mixture of methanol–water (1:9, v/v).

2.4. Liquid chromatography–mass spectrometry (LC–MS/MS)

The chromatographic separation was carried out using an Agilent 1260 UHPLC from Agilent Technologies (Waldbronn, Germany) with an automatic injector of 100 samples and a column Kinetex™ 1.7 μm XB-C18100A, with a length of 50 × 2.1 mm and manufactured by Phenomenex® at a temperature of 30 °C with a constant flow of 0.2 $mL\,min^{-1}$. The mobile phases were deionized water (A) and methanol (B) both with 0.1% of formic acid. The gradient elution started at 10% B maintained for 5 min, then increased linearly to 95% B in 12 min and remained constant at 95% B up to 25 min, then it returns to the initial conditions with an equilibrium time of 15 min. The injection volume was 5 μL. The chromatograph was attached to an Agilent 6410 Mass Spectrometer Triple quadrupole from Agilent Technologies (California, USA) equipped with an electrospray (ESI) source operating in positive ionization at a gas temperature of 300 °C, a gas flow of 11 $L\,min^{-1}$, and pressure of 25 psi and a voltage of 4000 V.

2.5. Method validation

The performance of the method was evaluated through linearity, sensitivity, recovery, precision, and matrix effect. Quantification was performed by the IS method. Deuterated compounds were added to the standard solutions and samples to get a concentration of 25 $\mu g\,L^{-1}$ of each drug in the final extract (25 μL of a mixture at 1 $mg\,L^{-1}$ was added to samples, extract or standard solutions to a final volume of 1 mL).

Calibration curves were obtained by injecting standard solutions prepared in methanol–water (1:9, v/v) and in extracts of sediment, sludges and particulate matters at nine concentrations from 0.1 $\mu g\,L^{-1}$ to 100 $\mu g\,L^{-1}$ (equivalent to 0.1 and 100 $ng\,g^{-1}$ d.w., respectively). The value of signal generated by each compound present in the sample blank was subtracted to each point of the matrix-matched calibration curve. Calibration curves were constructed by weighted ($1/x^2$) least squares linear regression of observed analyte-to-IS peak-area ratios against concentration.

The matrix effect were evaluated for sludge, sediment and particulate matter by comparing the peak area obtained for each compound in the final extract (A_{sample}) (after subtracting the amount of the analyte in the blank, if present (A_{blank})), and in a standard solution (A_{std}) at a concentration of 100 $\mu g\,L^{-1}$, equivalent to 100 $ng\,g^{-1}$ d.w. [25]. According to the following equation: $\{[(A_{sludge} - A_{blank})/A_{std}]\} \times 100$, if the value obtained is higher than 100, the analyte MS signal is enhanced by matrix components, whereas if the value obtained is lower than 100, the ionization of the analyte is being suppressed. Values close to 100 indicate the absence of matrix effect.

The limits of detection (LODs) and quantification (LOQs) of the method were experimentally estimated from the analysis of particulate matter, sludge and sediment samples as the concentration of analyte giving a signal-to-noise ratio (S/N) of 3 and 10, respectively. When the target compound was not detected in any of the analyzed samples, LODs and LOQs were obtained from the S/N values observed in the recovery study carried out at the lowest level of fortification (50 $ng\,g^{-1}$ d.w., $n = 5$).

Recoveries in particulate matter, sludge and sediment, were calculated by spiking the samples at 50 and 100 $ng\,g^{-1}$. The standard solution was added with a 100 μL gas chromatograph syringe to distribute it homogenously in the sample. The sample was left at room temperature for 15 min to ensure balance. Standard extraction was then performed to check the percentage recovered. Each recovery level was tested in quintuplicated. In addition, a non-spiked blank sample was extracted to correct the error due to the presences of drugs in the samples.

Precision was expressed by the relative standard deviation (RSD) of 5 replicated measures. Intra-day (repeatability) and inter-day (reproducibility) precision was determined by analyzing a concentration of 50 $ng\,g^{-1}$ d.w. on the same day or on 5 different days.

To ensure quality of the determination, some analyte stability studies were carried out. Analyte degradation in the samples frozen at −20 °C was tested analyzing the same sample weakly for three months. Drying step either lyophilization or overnight drying at room temperature was tested analyzing spiked samples. The environmental degradation takes place mostly in water by hydrolysis and photodegradation. The analytes were stable in the frozen samples at least three months and for drying processes degradation was <15%. This agrees with previous studies on the subject [16,18,20].

3. Results and discussion

3.1. Liquid chromatography–mass spectrometry

Prior to chromatographic separation, the MS/MS conditions were optimized to achieve the highest sensitivity for each analyte. The optimization was carried out using the optimizer program included in the software that checks the optimum fragmentor voltage for the precursor ion and the collision energy more appropriate for each product ion. These parameters were optimized by injecting each compound at 500 $\mu g\,L^{-1}$ without analytical column using an isocratic mobile phase of water–methanol (1:1, v/v), both with 0.1% of formic acid at a flow rate of 0.2 $mL\,min^{-1}$. This mobile phase was tentatively selected to optimize fragmentation considering that most of the target compounds have basic properties as

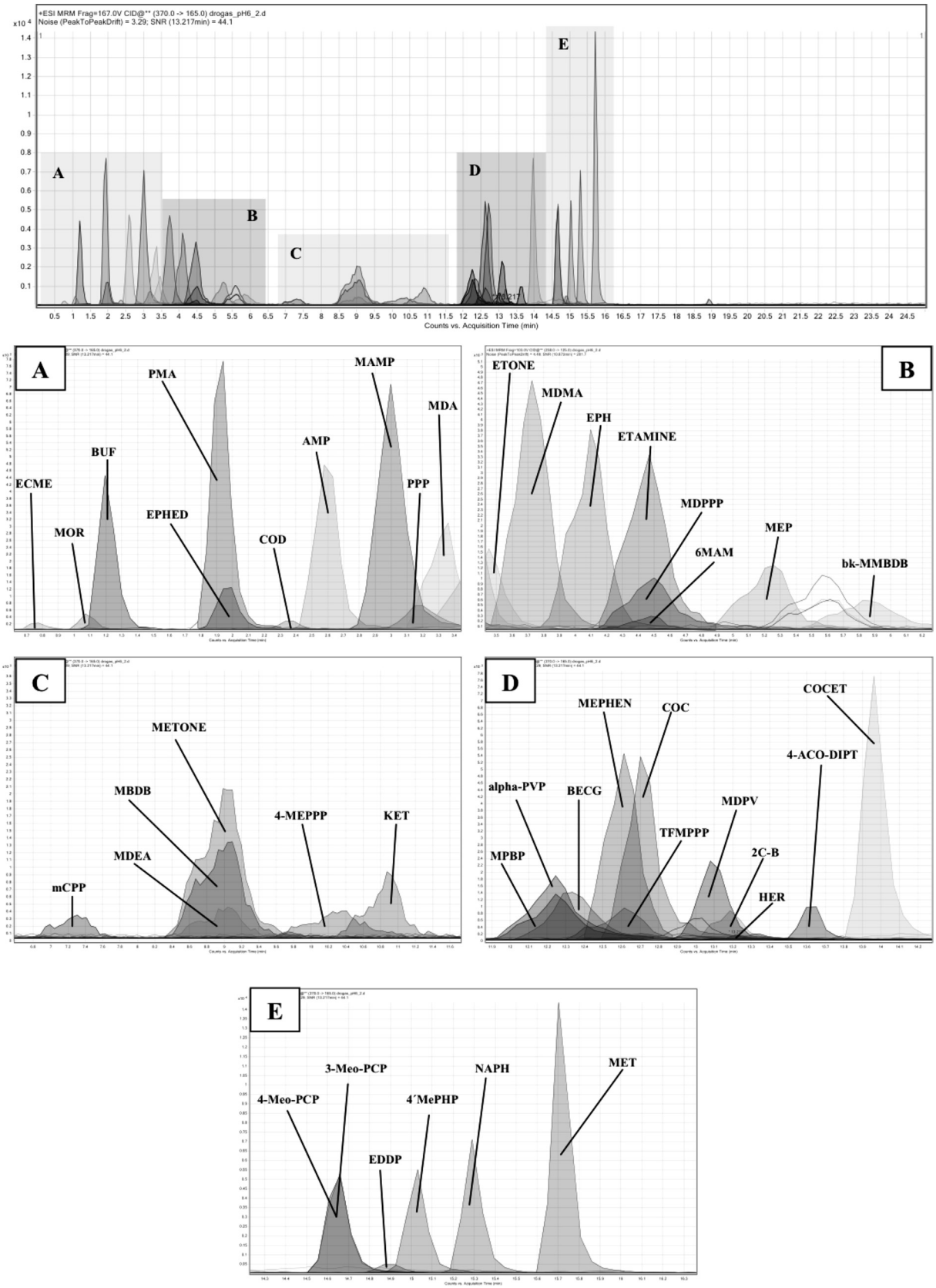

Fig. 2.1. Chromatograms of the illicit drugs analyzed in the current work at a concentration of 25 ng g^{-1}.

well as the mobile phases reported in some previous works for the most traditional drugs [12–19,26].

Regarding to the chromatographic separation, 41 compounds were separated (Fig. 2.1) in just 16 min due to the efficiency of the Kinetex C_{18} column with core-shell technology which can be leveraged to increase resolution, improve productivity and decreases solvent consumption. This column provided sharp chromatographic peaks for most analytes but not all. Fig. 2.1 shows

Table 2.2
Retention times and MS/MS parameters employed for the SRM acquisition.

Illicit drug	RT (min)	Fragmentor	Quantitative		Qualitative		RSDs of the ion ratio (%)
			SRM	Collision energy	SRM	Collision energy	
2C-B	13.19	88	260 → 243	5	260 → 228	21	13
PMA	1.83	74	166 → 148	9	166 → 121	21	15
AMP	2.53	74	136 → 91	13	136 → 65	41	5
MAMP	2.94	74	150 → 91	5	150 → 119	17	4
ETAMINE	4.47	88	164 → 91	9	164 → 119	17	16
MEPHEN	12.61	113	234 → 84	17	234 → 56	53	2
EPH	4.05	74	166 → 149	5	166 → 91	33	1
KET	10.87	108	238 → 125	25	238 → 89	73	1
3-MeO-PCP	14.66	76	274 → 189	10	274 → 86	10	11
4-MeO-PCP	14.66	78	274 → 189	4	274 → 121	26	5
EPHED	1.89	96	164 → 146	10	164 → 131	18	18
MEP	5.20	88	178 → 160	9	178 → 145	21	5
COC	12.75	123	304 → 182	17	304 → 82	29	8
ECME	0.72	113	200 → 182	17	200 → 82	25	19
COCET	13.96	132	318 → 196	17	318 → 82	29	19
BECG	12.34	123	290 → 168	17	290 → 105	29	2
COD	2.23	164	300 → 115	78	300 → 152	90	18
MOR	0.86	152	286 → 152	45	286 → 165	69	14
6-MAM	4.43	172	328 → 165	41	328 → 152	81	17
EDDP	14.92	166	279 → 250	20	279 → 235	20	17
MET	15.75	108	310 → 265	9	310 → 105	25	9
HER	13.22	167	370 → 58	53	370 → 165	25	8
PPP	3.20	128	204 → 105	14	204 → 133	30	4
MDPPP	4.45	136	248 → 98	26	248 → 91	46	3
4-MePPP	10.19	116	218 → 147	14	218 → 119	14	16
α-PVP	12.24	128	232 → 91	22	232 → 77	58	14
MPBP	13.03	128	232 → 91	14	232 → 161	34	9
4′ MePHP	15.03	128	260 → 105	18	260 → 91	50	16
MDA	3.31	74	180 → 163	5	180 → 105	21	1
ETONE	3.40	96	222 → 174	10	222 → 204	18	15
MDMA	3.67	88	194 → 163	10	194 → 77	50	6
bk-MMBDB	5.77	118	236 → 86	18	236 → 161	26	4
MBDB	8.93	93	208 → 135	17	208 → 51	77	9
METONE	8.93	93	208 → 135	29	208 → 77	49	10
MDEA	5.56	98	208 → 77	29	208 → 105	49	16
NAPH	15.29	137	282 → 141	25	282 → 77	85	5
MDPV	13.13	132	276 → 126	25	276 → 135	25	14
mCPP	7.25	123	197 → 154	25	197 → 91	61	10
TFMPP	12.61	123	231 → 188	21	231 → 44	21	11
4-AcO-DIPT	13.65	116	303 → 114	14	303 → 202	14	3
BUF	1.14	98	205 → 58	14	205 → 160	10	11

the chromatograms obtained in SRM mode corresponding to the LC–MS/MS analysis of a sludge sample spiked at 25 ng g^{-1}. MDPPP, 6-MAM, MEP, bk-MMBDB, mCPP, MDEA, MBDB, METONE, 4-MePPP, KET, MPBP and alpha-PVP provides wide peaks but still quantifiable.

Table 2.2 lists the retention time of the studied analytes under the optimum separation conditions. It also details the transitions selected for quantification and confirmation (qualitative). In order to evaluate the performance of the LC–MS/MS, linearity, instrumental detection limits (IDLs) and instrumental quantification limits (IQLs) were evaluated for each compound. Target analytes showed a good linear range ($r^2 \geq 0.99$) between 0.50 and 100 μg L^{-1}, with the lowest point in the calibration curve being considered as the IQLs (graphs obtained and equation can be seen in supplementary mate-rial, Fig. S2.1). The instrumental detection limits (IDLs), calculated as a signal-to-noise ratio (S/N) of 3, ranged from 0.001 to 0.32 μg L^{-1} for all the compounds.

3.2. Optimization of the extraction procedure

The extraction conditions (solvents, buffers, percentage of mixture, volume) as well as the clean-up conditions including sample pH, sample volume, washing step and elution solvent and volume were optimized to promote and enhance the extraction of the analytes from the solid matrices and their retention on the sorbents. These experiments were carried out by spiking in triplicate sediment samples at 100 ng g^{-1} of each analyte. Results were confirmed in the other two matrices.

3.2.1. Extraction solvent

The first parameter optimized was the extraction solvent, water, methanol and water–methanol mixture (1:1, v/v) were tested. The best results were obtained with the methanol–water mixture, which provided recoveries >35% for all the analytes with the excep-tion of ECME (3%) and EDDP (13%) (Table 2.3). These compounds are non-charged at the neutral pH so their extraction efficiency is low. Previous studies have reported the use of several acidi-fied water–methanol mixtures as the optimum extracting solvent [10,13,27,28]. A broad range of pH in the neutral and acidic region (2, 3.5, 4.5, 6.5, 8) was tested. The McIlvaine buffer was selected because varying its composition provided an approximately con-stant ionic strength (about 60 mM as calculated by an adapted Henderson-Hasselbalch equation) over a broad pH range with-out having to exchange the buffer system. The McIlvaine buffer has been used previously to extract pharmaceuticals from solid matrices, such as marine sediment [29]. The pH that provided the optimum results was 4.5. In Table 2.3, the results of extracting sediments with methanol–McIlvain buffer at pH 4.5 are shown. According to the results obtained using this method, the recovery was improved in the 70% of drugs tested. Furthermore there are some cases as ECME and EDDP have a positive charge with 4.5 pH of the McIlvaine buffer increasing their extraction. Although 30%

Table 2.3
Efficiency (recovery %) of extraction procedure (without or with McIlvine buffer) and different pH of the extract on the SPE. Distilled water was spiked at 100 ng L^{-1}.

Illicit drug	Recovery (%)					
	Extraction procedure		SPE clean-up			
	Without buffer	With buffer	pH 3	pH 4	pH 5	pH 6
2C-B	53	72	97	78	51	75
PMA	80	107	10	n.d.	67	93
AMP	68	95	18	23	79	87
MAMP	66	94	55	78	100	102
ETAMINE	79	56	73	87	84	90
MEPHEN	91	60	119	111	91	108
EPH	74	107	79	95	101	99
KET	69	105	142	179	105	106
3-MeO-PCP	88	68	89	101	96	98
4-MeO-PCP	83	65	85	92	89	90
EPHED	35	32	6	6	10	31
MEP	50	51	63	60	64	79
COC	95	108	114	112	92	113
ECME	3	75	0	10	30	40
COCET	95	106	88	94	81	88
BECG	102	93	76	87	102	87
COD	86	122	68	82	85	82
MOR	74	88	9	18	85	101
6-MAM	79	108	92	120	106	105
EDDP	13	64	99	98	84	87
MET	72	92	84	103	105	94
HER	87	107	41	27	21	62
PPP	75	58	64	77	58	68
MDPPP	76	106	103	107	98	92
4-MePPP	74	55	141	73	69	97
α-PVP	64	50	101	95	73	74
MPBP	60	52	97	93	78	83
4′ MePHP	68	57	102	108	77	86
MDA	76	55	63	79	87	88
ETONE	80	99	77	84	74	76
MDMA	87	110	116	137	144	105
bk-MMBDB	60	55	106	132	77	101
MBDB	97	123	114	122	119	114
METONE	97	122	114	122	119	114
MDEA	93	129	117	126	132	125
NAPH	58	101	69	75	62	69
MDPV	77	103	95	101	88	82
mCPP	53	115	98	99	69	79
TFMPP	59	99	85	82	69	71
4-AcO-DIPT	90	76	21	27	34	45
BUF	76	197	1	11	57	86

n.d. not detected.

of drugs, e.g. those derived from pyrrolidinophenones, presented worse recoveries than with unbuffered water. This occurs in a lesser extent (up to 23% of recovery reduction) in front of to 79% of recovery increasing with buffer. Therefore, the extraction was carried out at pH 4.5 with McIlvaine buffer–methanol (1:1, v/v) for sludge, sediments and particulate matter.

3.2.2. Optimization of SPE step

The pH of the extract can also influence the clean-up step since ionic and non-ionic interactions of the analytes with the solid sorbent playing a dominant role in the extraction. Some studies established that it is better to use basic pH in the case of the extraction of organic acids analytes in soils [30]. However, most studies carried out with drugs of abuse in water opted for an acid pH since it provides better recovery [10,13,19,29–31]. Based on these data, the effect of the pH of the extract on the SPE recoveries was evaluated by loading 245 mL of distilled water (plus 5 mL of methanol) spiked at a final concentration in the extract of 100 μg L^{-1} with the analytes and adjusted at different pH values (3, 4, 5 and 6). The differences between the recoveries obtained at the various pH are shown in Table 2.3. The influence of pH on the recoveries depends on the chemical structure of the drugs, thus, an agreement should be reached. Pyrrolidinophenone derivatives, piperazines and some entactogens, cocainics and opioids (Table S2.1) are better extracted at pH 3, while most of the remaining drugs are better extracted in less acidic environment. This situation is clearly depicted in Fig. 2.2. The compounds with shorter retention time (Fig. 2.2A) are better extracted at neutral pH, while compounds with longer retention time (Fig. 2.2B) are better extracted at acid pH. Overall extractions at neutral pH are better because some analytes as ECME and EDDP were degraded at pH 3 which is a quite extreme pH, so it is coun-terproductive for these compounds as already reported [SPE and LC–MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (Valencia, Spain) [32]. Therefore, pH 6 was selected and the extracts were adjusted with NaOH 1 M. Regarding the elution step different conditions were tested: methanol (6, 10 and 12 mL), 6 mL of methanol followed by 3 mL methanol with 1% formic acid and 6 mL of methanol followed by 3 mL of a methanol–dichloromethane solution (1:1, v/v). The best results were obtained with 6 mL of methanol followed by 3 mL of a methanol–dichloromethane solution (1:1, v/v). Acidified methanol elute better some compounds as EPH, AMP, MAMP and HER [16]. However, methanol-dichloromethane (1:1, v/v) after pure methanol also helped to increase the recovery of these compounds and was the preferred option to preserve ECME and EDDP. This SPE approach allowed recoveries between 62% (HER) and 125% (MDEA) for all analytes except the EPHED, ECME and 4-AcO-DIPT for which only 31%, 40% and 45% could be recovered. RSD values for all analytes were below 22%.

3.2.3. Method validation

The performance of the method was evaluated through linearity, sensitivity, recovery, precision, and matrix effects as described in Section 2.5. Method performance data are provided in Tables 2.4 and 2.5. Response of the MS signal between 0.1 μg L^{-1} or the analyte limit of quantification if higher and 100 μ g L^{-1} (equivalent to 0.1 ng g^{-1} d.w. and 100 ng g^{-1} d.w.) was linear for all analytes, obtaining $r^2 \geq 0.99$ with nine points. The good linearity of each compound including equations is shown in the supplementary material (see Fig. S2.1). Many target compounds were subject to matrix ionization suppression or enhancement effects. As shown in Table 2.4 for sediments, only MEP, 6-MAM, MET, MDA, METONE, mCPP, PMA and α -PVP gave a response around 100%. Previous studies already pointed out that the lowest drop in the analytical signal was observed for amphetamine-like compounds [16]. Fifteen compounds gave signal enhancement and the others present a sup-pression signal ranging from 41% (KET) to 89% (bk-MMBDB). Again, considering the high matrix effect observed in the analysis of this type of samples, the use of isotopically labeled IS for quantification is crucial.

LODs and LOQs were lower than 1.32 and 3.96 ng g^{-1} d.w., respectively, in all the matrices. Table 2.4 also outlines LOD and LOQ for sediments. The results in sludges and particulate matter were similar (data not shown). These values are in agreement with those reported in the other methods developed (Table 2.1). Cocainics showed the lowest LODs and LOQs and MDA, MDPV and mCPP the highest. Recoveries of the overall method were calculated by analyzing sediments, sludges and particulate matter samples (n = 5 for each) fortified at two concentration levels (50 and 100 ng g^{-1} d.w.). Table 2.5 shows the relative recoveries of the method at 50 ng g^{-1} d.w. Relative recoveries ranged from 25 to 94% for particulate matter, from 19 to 97% for sludge and between 14 and 109% for sediments. The low recoveries and the nature of such a complex matrix require the use of isotopically labeled analogs as surrogate standards, in order to achieve reliable results.

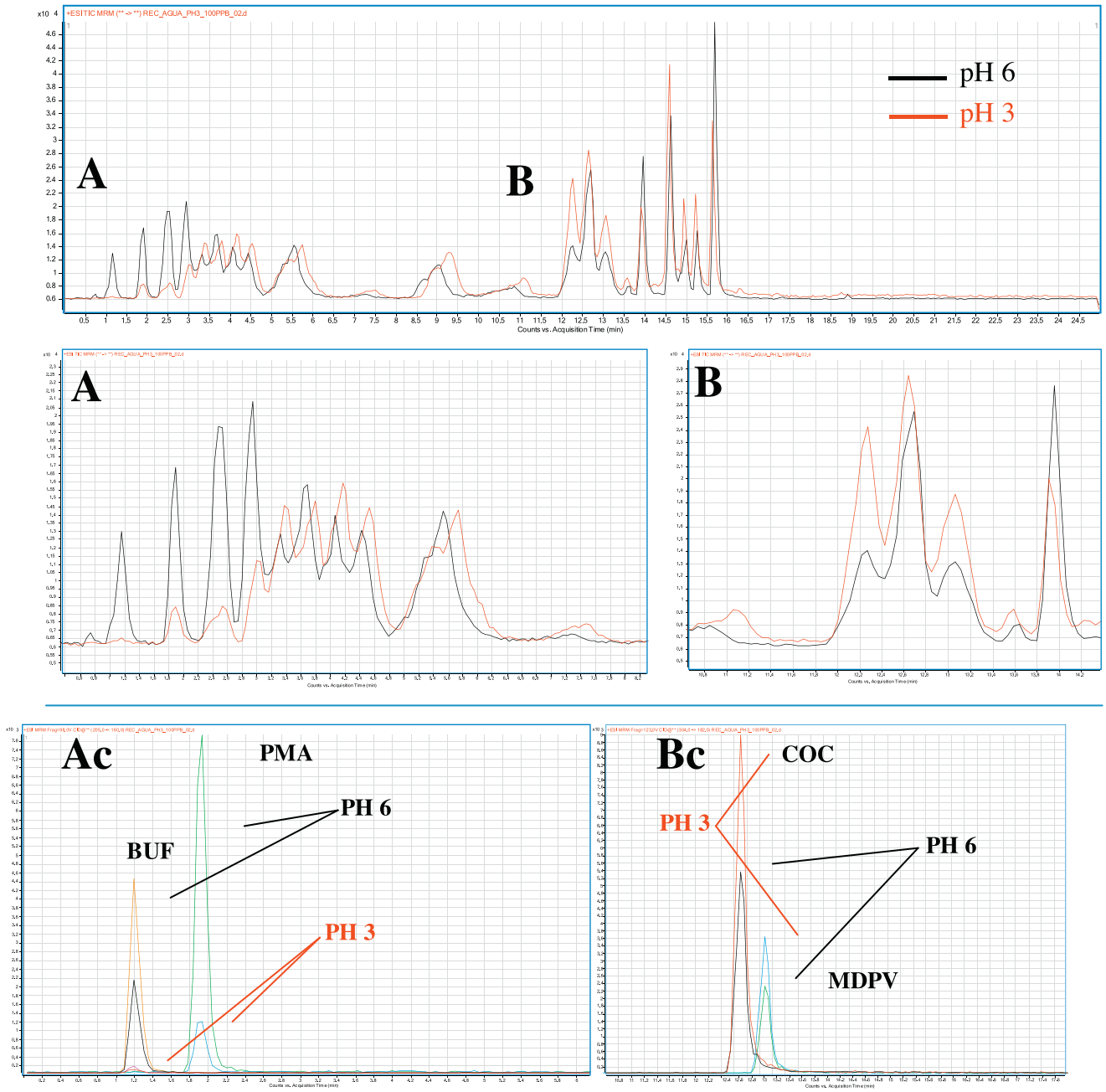

Fig. 2.2. Chromatograms of the extractions with pH 3 and pH 6. Ac and Bc shows examples of concrete compounds: (Ac) Chromatograms of PMA and BUF, (Bc) Chromatograms of COC and MDPV.

3.3. Application

A total of 29 sludge samples, 5 sediments and 50 particulate matter samples were analyzed. The detailed results in each individual samples are outlined in Figs. 2.3–2.5, Table 2.6 and in the supplementary material (Tables S2.2–S2.7).

3.3.1. Drugs tested in particulate matter samples

The results of the quantification of drugs in particulate matter from wastewater influent of the three WWTPs are shown in Fig. 2.3 and Table 2.6. Cocainics and morphine derivatives were the most frequently detected. BUF was scarcely detected in a few samples at concentrations ranging between 22 and 63 ng g^{-1} and KET was detected only in one at a concentration of 46 ng g^{-1}.

Pinedo I, presents high concentrations of COC and its metabolite ECME, while the metabolite COCET was only detected in one sample, whereas BECG, the main metabolite of the COC was absent. On the contrary in the case of Pinedo II and Quart-Benàger, BECG was the most frequent drug detected in a concentration up to 223.1 ng g^{-1} and 119.9 ng g^{-1} respectively. Sporadic concentrations of COC in Pinedo II and COCET in Quart-Benàger were observed. These compounds present a recreational use because their highest values were detected at weekends. High values during weekdays on the second week may be due to the celebration of Fal-las Festivity that takes place between 15th and 19th March every year. Some studies have demonstrated this temporal consumption trend in influents wastewater samples [26]. The morphine deriva-tives, MET and COD, were also detected in Pinedo I and II but not in Quart-Benàger. MET was detected in almost all samples at variable

Table 2.4
Repeatability, reproducibility, LOD, LOQ and matrix effect in sediment samples.

Illicit drug	Repeatability (RSDs, %)	Reproducibility (RSD, %)	LOD ($ng\,g^{-1}$)	LOQ ($ng\,g^{-1}$)	Matrix effect (%)
2C-B	2	6	0.12	0.36	140
PMA	1	8	0.73	2.19	91
AMP	6	5	0.51	1.53	77
MAMP	2	13	0.22	0.66	55
ETAMINE	3	8	0.41	1.23	75
MEPHEN	5	8	0.15	0.45	129
EPH	3	6	0.95	2.85	192
KET	5	15	0.15	0.45	41
3-MeO-PCP	5	19	0.21	0.63	46
4-MeO-PCP	7	19	0.23	0.69	45
EPHED	5	9	0.87	2.61	225
MEP	3	20	0.76	2.28	105
COC	20	20	0.05	0.15	192
ECME	4	20	0.77	2.31	135
COCET	19	15	0.10	0.30	120
BECG	3	8	0.04	0.12	120
COD	3	21	0.32	0.96	188
MOR	3	12	0.17	0.51	150
6-MAM	14	6	1.10	3.30	104
EDDP	10	7	1.12	3.36	80
MET	2	3	0.25	0.75	98
HER	4	6	0.87	2.61	165
PPP	5	3	0.26	0.78	125
MDPPP	5	10	0.43	1.29	84
4-MePPP	4	19	0.87	2.61	71
α-PVP	6	14	0.21	0.63	109
MPBP	5	14	0.36	1.08	52
4′-MePHP	2	17	0.35	1.05	51
MDA	5	16	1.12	3.36	103
ETONE	4	9	0.39	1.17	170
MDMA	1	2	0.37	1.11	65
bk-MMBDB	3	7	0.34	1.02	89
MBDB	3	13	0.74	2.22	79
METONE	8	19	0.81	2.43	109
MDEA	3	12	0.90	2.70	60
NAPH	7	9	0.24	0.72	89
MDPV	2	5	1.32	3.96	78
mCPP	2	9	1.30	3.90	103
TFMPP	4	19	0.43	1.29	120
4-AcO-DIPT	5	16	0.98	2.94	167
BUF	2	18	0.21	0.63	59

concentrations that did not present a clear pattern. In the case of COD, its presence is sporadic in a few samples. The constant presence of MET may be due to the continuous controlled delivery of this compound to the population to treat addiction to opiates (such as HER).

3.3.2. Drugs testing in sludges

A wide variety of drugs have been detected in sludges, including cocainics, some morphine derivatives, arylcyclohexylamines, hallucinogens and BUF. Regarding cocainicis (Fig. 2.4), COC con-centrations were higher than its metabolites BECG and ECME, probably because its metabolites are more easily degraded than unaltered COC. Values of these compounds determined in Pinedo were slightly higher than those detected in Quart-Benàger. The data does not show any temporal trend, probably, because sludge of several days can be mixed.

Table 2.5
Relative recoveries (R (%) ± RSDs, $n = 5$) in the different solid abiotic matrices calculated at a concentration of 50 $ng\,g^{-1}$ d.w.

Illicit drug	R (%) ± RSDs, $n = 5$		
	Particulate matter	Sediments	Sludges
2C-B	61 ± 18	56 ± 6	60 ± 23
PMA	94 ± 19	89 ± 9	75 ± 2
AMP	79 ± 13	72 ± 18	68 ± 14
MAMP	68 ± 9	66 ± 11	59 ± 17
ETAMINE	76 ± 20	85 ± 11	73 ± 9
MEPHEN	55 ± 11	70 ± 3	60 ± 20
EPH	63 ± 14	55 ± 31	53 ± 13
KET	81 ± 13	78 ± 8	70 ± 14
3-MeO-PCP	92 ± 13	80 ± 5	72 ± 17
4-MeO-PCP	94 ± 9	90 ± 4	82 ± 30
EPHED	25 ± 19	25 ± 19	19 ± 25
MEP	53 ± 9	60 ± 7	51 ± 12
COC	61 ± 19	77 ± 18	50 ± 16
ECME	25 ± 20	30 ± 22	19 ± 24
COCET	83 ± 3	109 ± 8	70 ± 16
BECG	77 ± 2	106 ± 12	75 ± 4
COD	78 ± 10	70 ± 10	68 ± 16
MOR	94 ± 12	102 ± 26	97 ± 10
6-MAM	85 ± 14	97 ± 21	63 ± 18
EDDP	47 ± 23	52 ± 19	29 ± 24
MET	84 ± 6	14 ± 10	83 ± 21
HER	35 ± 19	34 ± 18	25 ± 16
PPP	50 ± 16	62 ± 10	57 ± 6
MDPPP	67 ± 20	83 ± 9	60 ± 13
4-MePPP	74 ± 15	60 ± 12	57 ± 19
α-PVP	63 ± 12	58 ± 17	50 ± 13
MPBP	64 ± 12	52 ± 7	54 ± 20
4′-MePHP	74 ± 20	67 ± 20	52 ± 18
MDA	41 ± 10	55 ± 17	55 ± 14
ETONE	82 ± 10	97 ± 9	77 ± 5
MDMA	82 ± 10	88 ± 4	51 ± 10
bk-MMBDB	51 ± 23	87 ± 9	51 ± 11
MBDB	72 ± 11	62 ± 9	52 ± 13
METONE	84 ± 17	75 ± 3	51 ± 17
MDEA	86 ± 10	59 ± 6	54 ± 9
NAPH	79 ± 11	66 ± 17	56 ± 16
MDPV	80 ± 16	80 ± 18	87 ± 36
mCPP	68 ± 17	64 ± 6	89 ± 19
TFMPP	55 ± 19	68 ± 15	86 ± 18
4-AcO-DIPT	36 ± 22	31 ± 24	25 ± 25
BUF	90 ± 15	82 ± 15	64 ± 10

Table 2.6
Summary of the results obtained in particulate matter, sewage sludges and sediments.

Illicit drug	Concentration ($ng\,g^{-1}$ d.w.)								
	Particulate matter			Sludges			Sediments		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
PMA	n.d.	n.d.	n.d.	8	40	17	n.d.	n.d.	n.d.
KET	46	46	46	2	3	2	n.d.	n.d.	n.d.
4-MeO-PCP	n.d.	n.d.	n.d.	1	1	1	1	1	1
COC	14	127	49	4	58	26	30	30	30
ECME	14	143	77	7	9	8	n.d.	n.d.	n.d.
COCET	6	51	31	1	1	1	n.d.	n.d.	n.d.
BECG	3	223	80	1	4	2	1	1	1
COD	53	318	142	8	78	23	n.d.	n.d.	n.d.
MOR	n.d.	n.d.	n.d.	24	171	72	n.d.	n.d.	n.d.
MET	21	207	99	2	21	11	1	1	1
BUF	22	63	45	2	42	11	n.d.	n.d.	n.d.

n.d. not detected.

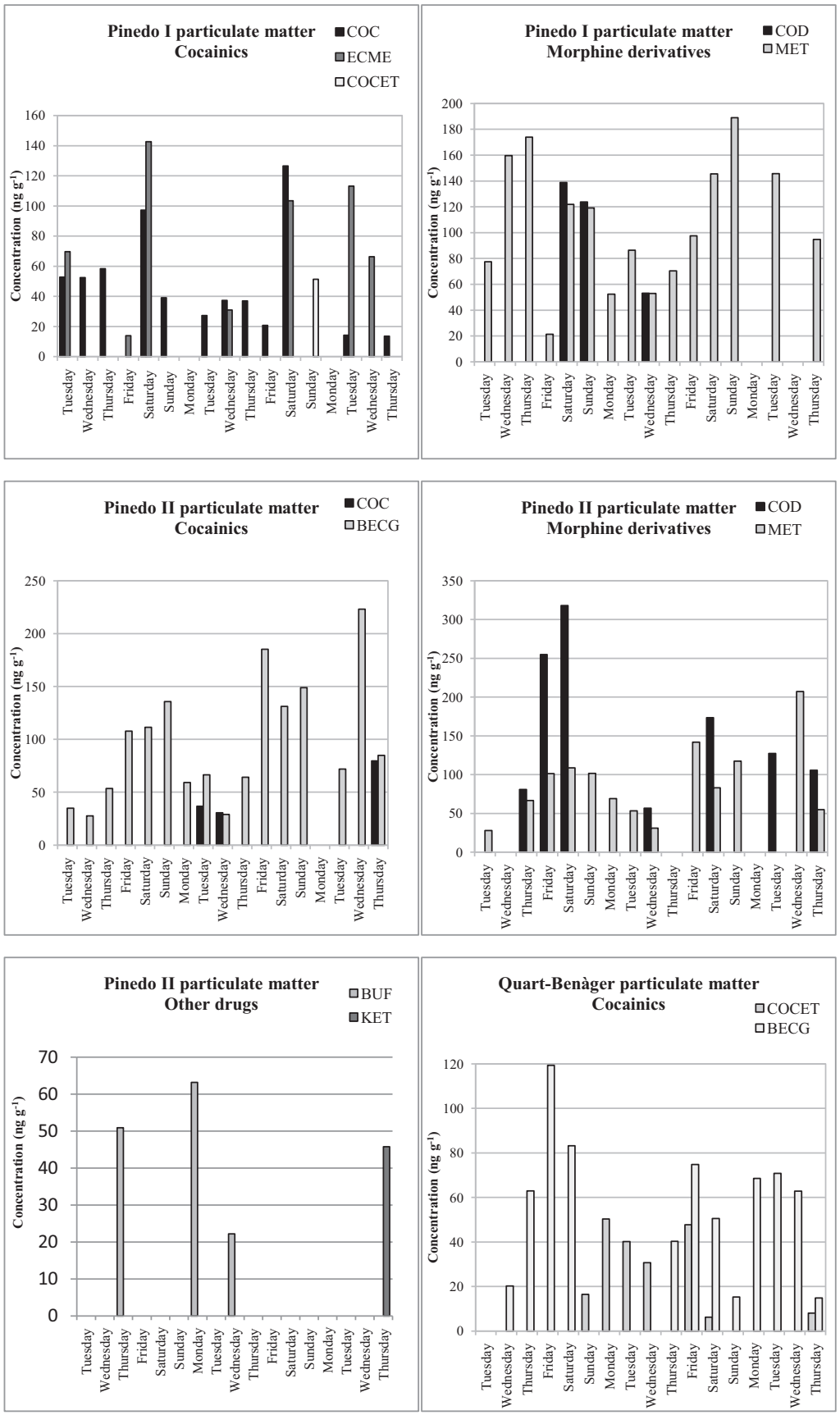

Fig. 2.3. Different illicit drugs concentration in influents of particulate matter of the studied WWTPs taken from 4th to 20th March 2014.

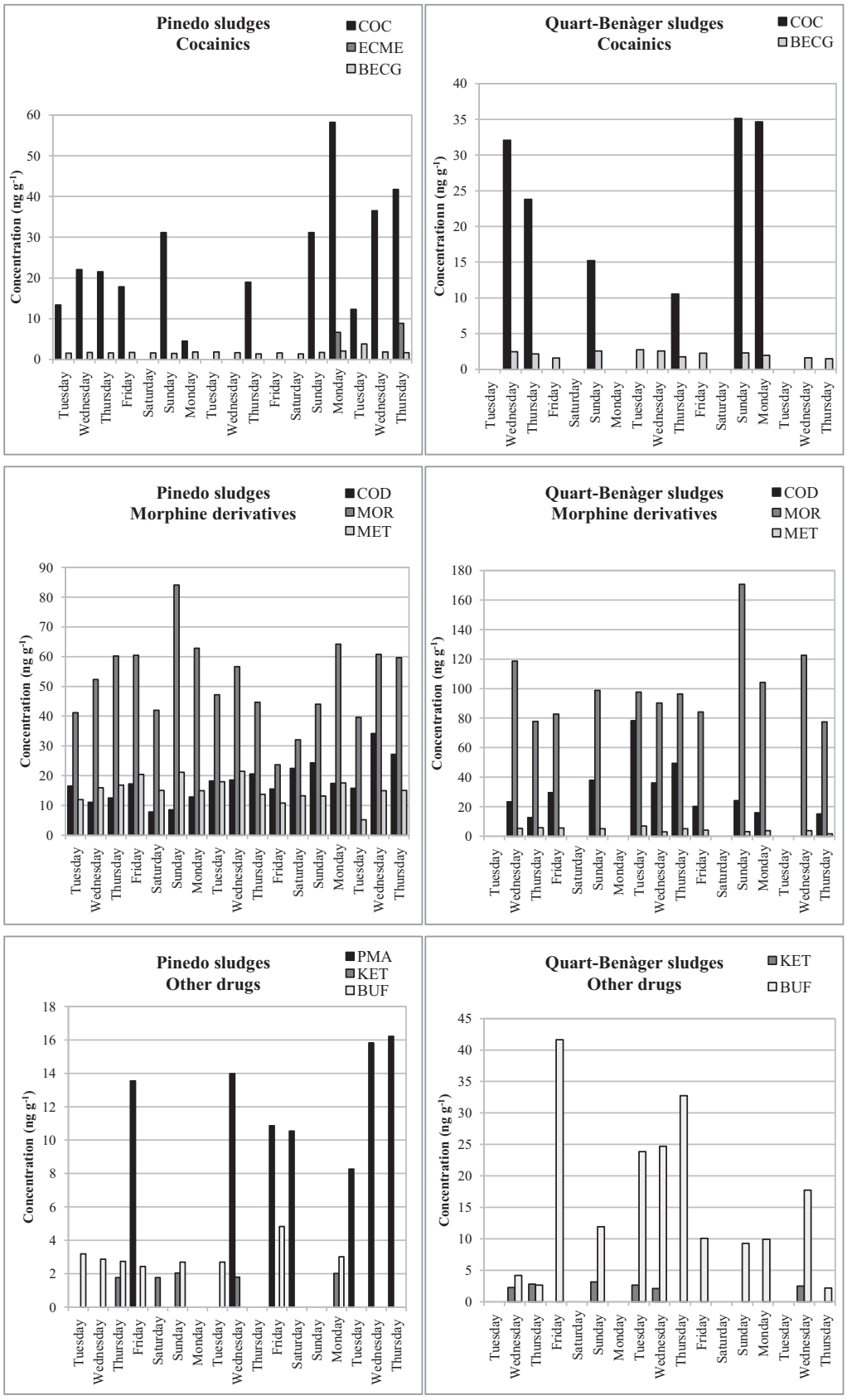

Fig. 2.4. Different illicit drugs concentration in sewage sludge of the studied WWTPs taken from 4th to 20th March 2014.

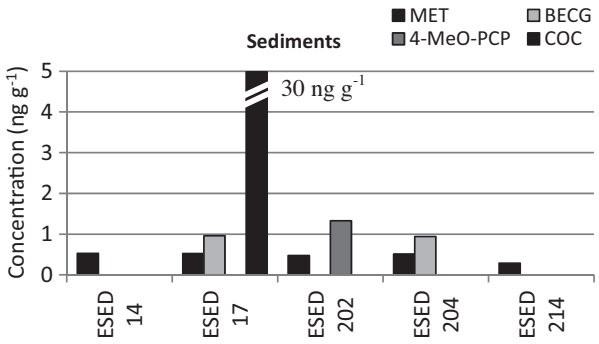

Fig. 2.5. Different illicit drugs concentration in Turia river sediments.

Morphine derivatives were found in higher concentrations than cocainics (Fig. 2.4). Morphine derivatives were detected in Pinedo and Quart-Benàger samples. MOR, MET and COD were the most abundant. PMA, KET and BUF appear at low concentrations. These dehydrated sludges are usually used as fertilizer on farmland, so the illicit drugs that these sludges contain could contaminate the substrate and percolate into groundwater. It could also affect the germination and growth of cultivated plants as has been shown to occur in the case of oat (*Avena sativa*) and lettuce (*Lactuca sativa*)[34]. In general, the use of substances from sewage for agricultural purposes is discouraged [33,34].

3.3.3. Drug testing in sediments

Only 5 sediments were analyzed by the presence of drugs of abuse, appearing two cocainics (COC and BECG), a derivative of morphine (MET) and one arylcyclohexylamine (4-MeO-PCP). COC and 4-MeO-PCP were in one sample at a concentration of 30 and 1.33 ng g^{-1}, respectively. MET were detected in all samples at a concentration ranging from 0.29 to 0.53 ng g^{-1} and BECG were detected in 2 samples in a concentration of 0.94 and 0.96 ng g^{-1} as shown in Fig. 2.5. Except for the COC, the concentrations were low, so do not suppose a risk to the fauna and flora from the river. The presence of these drugs probably is because the river water was polluted and sediment absorbed them.

4. Conclusion

A multi-residue methodology based on SLE, SPE clean-up and LC–MS/MS analysis was developed for the determination of highly abused illicit drugs and some of their metabolites in particulate matter, sewage sludge and sediments. 21 of the 41 target compounds are investigated in these environmental solid matrices for the first time. In spite of the complexity of the nature of these samples, reliable determination of the target compounds is possible thanks to the use of isotopically labeled analogs as surrogate standards.

The method developed for particulate matter, sewage sludge and sediments is suitable for the determination of these compounds, providing recovery between >50% for most of the compounds, LOQs under 3.96 ng g^{-1} for all compounds and reproducibility <20%.

In the suspended particles only derivatives of COC and opioids such as COC, ECME, COCET, MET and COD were detected. The results show a clear recreational use of cocainics whose consumption increases during the weekend and during Fallas festivity. In sewage sludge higher levels of COC than their metabolites were found with a constant presence throughout the entire study. The opioids – MOR, COD and MET – were at a higher concentration than cocainics. Some new psychoactive drugs as PMA, KET and BUF were detected, although their presence was still low. The incidence of drug abuse in sediments is scarce, but COC, 4-MeO-PCP, MET and BECG were detected. Only the MET was found in all samples and COC, but only occasionally was found in high concentrations (30 ng g^{-1}).

Acknowledgements

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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.2015.05.062

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SUPPLEMENTARY MATERIAL

Simultaneous determination of traditional and emerging illicit drugs in sediments, sludges and particulate matter

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Table S2.1
Information on drugs analyzed in this study.

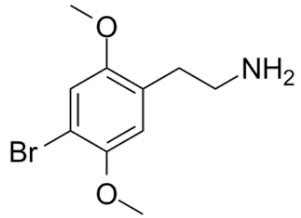
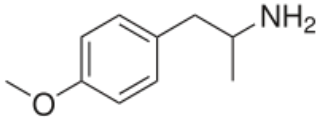
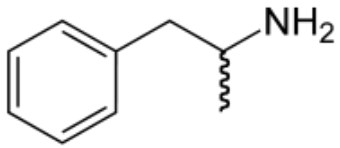
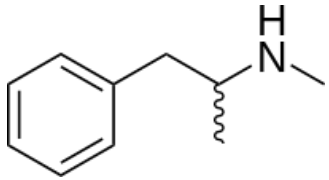
Name	Classification	Formula	Features	Deuterated
4-Bromo-2,5-dimethoxyphenethylamine (2C-B)	Family: Hallucinogens Effect: Hallucinogen Age: Traditional		IUPAC Name: 2-(4-bromo-2,5-dimethoxyphenyl)ethanamine Formula: $C_{10}H_{14}BrNO_2$ Mw: 260,13 g/mol	-----
4-methoxyamphetamine (PMA)	Family: Hallucinogens Effect: Hallucinogen Age: Traditional		IUPAC Name: 1-(4-methoxyphenyl)propan-2-amine Formula: $C_{10}H_{15}NO$ Mw: 165,23 g/mol	-----
Amphetamine (AMP)	Family: Amphetamines Effect: Psychostimulant Age: Traditional		IUPAC Name: (2S)-1-phenylpropan-2-amine + (2R)-1-phenylpropan-2-amine Formula: $C_9H_{13}N$ Mw: 135,2084 g/mol	D5: $C_9H_8D_5N$
Methamphetamine (MAMP)	Family: Amphetamines Effect: Psychostimulant Age: Traditional		IUPAC Name: N-methyl-1-phenylpropan-2-amine Formula: $C_{10}H_{15}N$ Mw: 149,23 g/mol	D5: $C_{10}H_{10}D_5N$

Table S2.1
Information on drugs analyzed in this study.

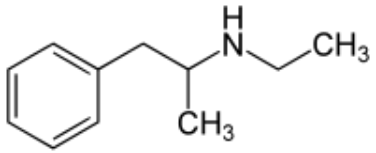
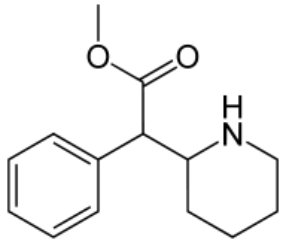
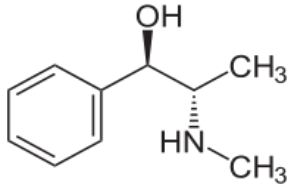
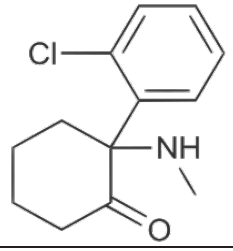
Name	Classification	Formula	Features	Deuterated
Ethylamphetamine (ETAMINE)	Family: Amphetamines Effect: Stimulant Age: New		IUPAC Name: N-ethyl-1-phenylpropan-2-amine Formula: $C_{11}H_{17}N$ Mw: 163,25 g/mol	-----
Methylphenidate (MEPHEN)	Family: Amphetamines Effect: Psychostimulant Age: Traditional		IUPAC Name: methyl 2-phenyl-2-piperidin-2-ylacetate Formula: $C_{14}H_{19}NO_2$ Mw: 233,30 g/mol	-----
Ephedrine (EPH)	Family: Amphetamines Effect: Psychostimulant Age: Traditional		IUPAC Name: 2-(methylamino)-1-phenylpropan-1-ol Formula: $C_{10}H_{15}NO$ Mw: 165,23 g/mol	-----
Ketamine (KET)	Family: Arylcyclohexylamines Effect: Hallucinogen and depressant Age: Traditional		IUPAC Name: 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one Formula: $C_{13}H_{16}ClNO$ Mw: 237,72 g/mol	D4: $C_{13}H_{12}D_4ClNO$

Table S2.1
Information on drugs analyzed in this study.

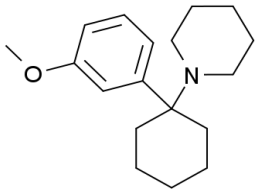
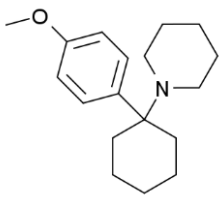
Name	Classification	Formula	Features	Deuterated
3-methoxyphencyclidine (3-MeO-PCP)	Family: Arylcyclohexylamines Effect: Depressant and hallucinogen Age: New		IUPAC Name: 1-[1-(3-methoxyphenyl)cyclohexyl]piperidine Formula: $C_{18}H_{27}NO$ Mw: 273,41 g/mol	-----
4-methoxyphencyclidine (4-MeO-PCP)	Family: Arylcyclohexylamines Effect: Depressant and hallucinogen Age: New		IUPAC Name: 1-[1-(4-methoxyphenyl)cyclohexyl]-piperidine Formula: $C_{18}H_{27}NO$ Mw: 273,41 g/mol	-----

Table S2.1
Information on drugs analyzed in this study.

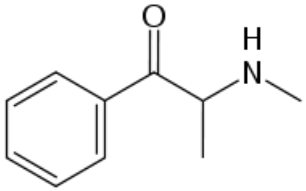
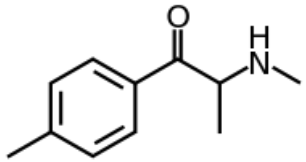
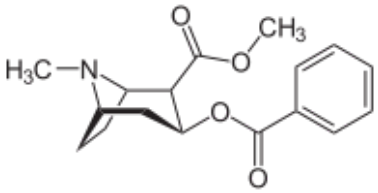
Name	Classification	Formula	Features	Deuterated
Ephedrone (EPHED)	Family: Cathinones Effect: Psychostimulant Age: Traditional		IUPAC Name: 2-(methylamino)-1-phenylpropan-1-one Formula: $C_{10}H_{13}NO$ Mw: 163,22 g/mol	-----
Mephedrone (MEP)	Family: Cathinones Effect: Psychostimulant Age: New		IUPAC Name: 2-(methylamino)-1-(4-methylphenyl)propan-1-one Formula: $C_{11}H_{15}NO$ Mw: 177,24 g/mol	-----
Cocaine (COC)	Family: Cocainics Effect: Stimulant Age: Traditional		IUPAC Name: methyl-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate Formula: $C_{17}H_{21}NO_4$ Mw: 303,35g/mol	D3: $C_{17}H_{18}D_3NO_4$

Table S2.1
Information on drugs analyzed in this study.

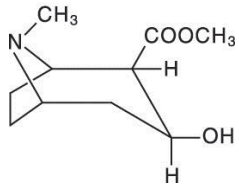
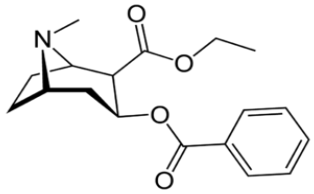
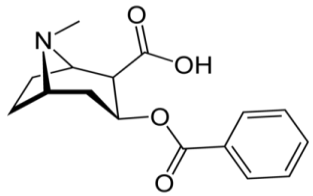
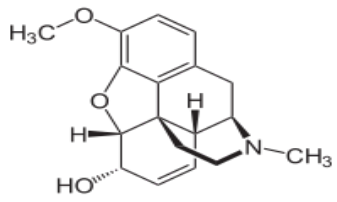
Name	Classification	Formula	Features	Deuterated
Ecgonine methyl ester (ECME)	Family: Cocainics Effect: Secondary metabolite		IUPAC Name: methyl 3-hydroxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate Formula: $C_{10}H_{17}NO_3$ Mw: 199,24 g/mol	D3: $C_{10}H_{14}D_3NO_3$
Cocaethylene (COCET)	Family: Cocainics Effect: Secondary metabolite		IUPAC Name: ethyl 3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate Formula: $C_{18}H_{23}NO_4$ Mw: 317,38 g/mol	D3: $C_{18}H_{20}D_3NO_4$
Benzoylecgonine (BECG)	Family: Cocainics Effect: Secondary metabolite		IUPAC Name: 3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylic acid Formula: $C_{16}H_{19}NO_4$ Mw: 289,33g/mol	D3: $C_{16}H_{16}D_3NO_4$
Codeine (COD)	Family: Morphine derivatives Effect: Depressant Age: New		IUPAC Name: (4R,4aR,7S,7aR,12bS)-9-methoxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7-ol Formula: $C_{18}H_{21}NO_3$ Mw: 299,36g/mol	-----

Table S2.1
Information on drugs analyzed in this study.

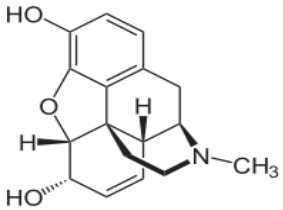
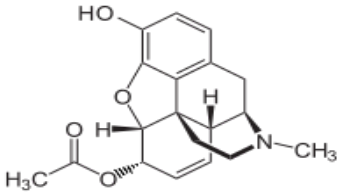
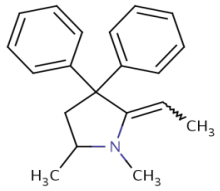
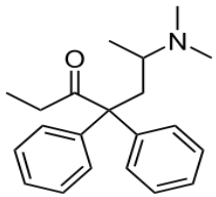
Name	Classification	Formula	Features	Deuterated
Morphine (MOR)	Family: Morphine derivatives Effect: Depressant Age: Traditional		IUPAC Name: 3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol Formula: $C_{17}H_{19}NO_3$ Mw: 285,34 g/mol	D3: $C_{17}H_{16}D_3NO_3$
6-monoacetylmorphine (6-MAM)	Family: Morphine derivatives Effect: Secondary metabolite		IUPAC Name: [(4R,4aR,7S,7aR,12bS)-9-hydroxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7-yl] acetate Formula: $C_{19}H_{21}NO_4$ Mw: 327,37 g/mol	D3: $C_{19}H_{18}NO_4D_3$
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	Family: Morphine derivatives Effect: Secondary metabolite		IUPAC Name: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrodine Formula: $C_{20}H_{23}N$ Mw: 277,40 g/mol	D3: $C_{20}H_{20}D_3N$
Methadone (MET)	Family: Morphine derivatives Effect: Depressant Age: Traditional		IUPAC Name: 6-(dimethylamino)-4,4-diphenylheptan-3-one Formula: $C_{21}H_{27}NO$ Mw: 309,44 g/mol	D3: $C_{21}H_{24}D_3NO$

Table S2.1
Information on drugs analyzed in this study.

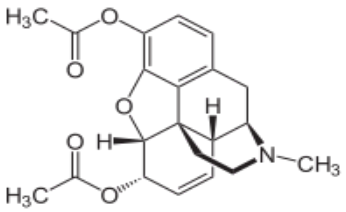
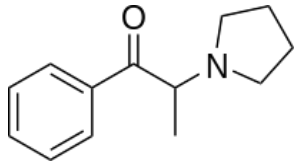
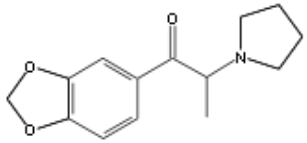
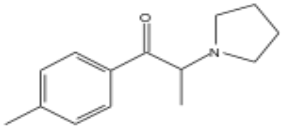
Name	Classification	Formula	Features	Deuterated
Heroin (HER)	Family: Morphine derivatives Effect: Depressant Age: Traditional		IUPAC Name: 9-acetyloxy-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7-yl] acetate Formula: $C_{21}H_{23}NO_5$ Mw: 369,41 g/mol	D9: $C_{21}H_{14}D_9NO_5$
α-pyrrolidinopropiophenone (PPP)	Family: Pyrrolidinophenone derivatives Effect: Stimulant Age: New		IUPAC Name: 1-phenyl-3-pyrrolidin-1-ylpropan-1-one Formula: $C_{13}H_{17}NO$ Mw: 203,28 g/mol	-----
3',4'-Methylenedioxy-alpha-pyrrolidinopropiophenone (MDPPP)	Family: Pyrrolidinophenone derivatives Effect: Stimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-2-pyrrolidin-1-ylpropan-1-one Formula: $C_{14}H_{17}NO_3$ Mw: 247,28 g/mol	-----
4-methyl-α-pyrrolidinopropiophenone (4-MePPP)	Family: Pyrrolidinophenone derivatives Effect: Stimulant Age: New		IUPAC Name: 1-(4-methylphenyl)-2-pyrrolidin-1-ylpropan-1-one Formula: $C_{14}H_{19}NO$ Mw: 217,31 g/mol	-----

Table S2.1
Information on drugs analyzed in this study.

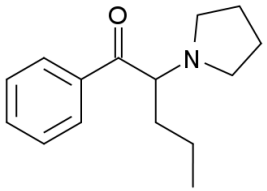
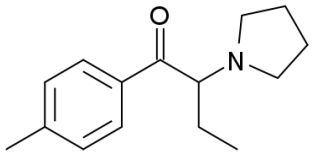
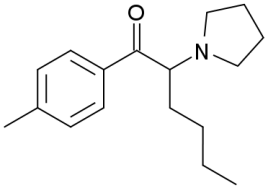
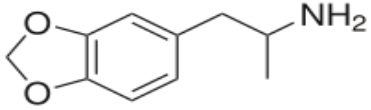
Name	Classification	Formula	Features	Deuterated
α-pyrrolidinopentiophenone (α -PVP)	Family: Pyrrolidinophenone derivatives Effect: Stimulant Age: New		IUPAC Name: 1-phenyl-2-pyrrolidin-1-ylpentan-1-one Formula: $C_{15}H_{21}NO$ Mw: 231,33 g/mol	-----
4-methyl-α-pyrrolidinobutiophenone (MPBP)	Family: Pyrrolidinophenone derivatives Effect: Stimulant Age: New		IUPAC Name: 1-(4-methylphenyl)-2-(pyrrolidin-1-yl)butan-1-one Formula: $C_{15}H_{21}NO$ Mw: 231,33 g/mol	-----
4-methyl-α-pyrrolidinohexaphenone (4'-MePHP)	Family: Pyrrolidinophenone derivatives Effect: Stimulant Age: New		IUPAC Name: 1-(4-methylphenyl)-2-pyrrolidin-1-ylhexan-1-one Formula: $C_{17}H_{25}NO$ Mw: 259,38 g/mol	-----
3,4-methylendoioxyamphetamine (MDA)	Family: Entactógenos Effect: Stimulant Age: Traditional		IUPAC Name: 1-(1,3-benzodioxol-5-yl)propan-2-amine Formula: $C_{10}H_{13}NO_2$ Mw: 179,22 g/mol	D5: $C_{10}H_8D_5NO_2$

Table S2.1
Information on drugs analyzed in this study.

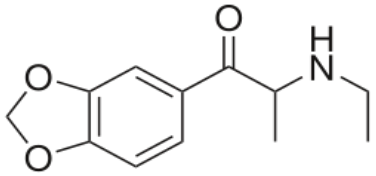
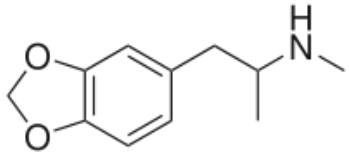
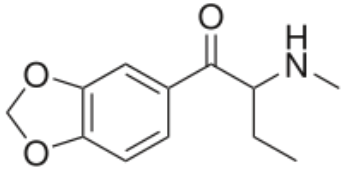
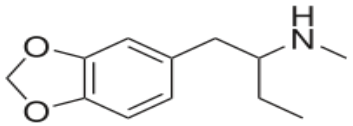
Name	Classification	Formula	Features	Deuterated
Ethylone (ETONE)	Family: Entactogens Effect: Stimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-2-(ethylamino)propan-1-one Formula: $C_{12}H_{15}NO_3$ Mw: 221.25 g/mol	-----
3,4-methylenedioxymethamphetamine (MDMA)	Family: Entactogens Effect: Stimulant Age: Traditional		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-N-methylpropan-2-amine Formula: $C_{11}H_{15}NO_2$ Mw: 193,25 g/mol	D5: $C_{11}H_{10}D_5NO_2$
Dibutylone (bk-MMBDB)	Family: Entactogens Effect: Stimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-2-(dimethylamino)butan-1-one Formula: $C_{13}H_{17}NO_3$ Mw: 235,28 g/mol	-----
N-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB)	Family: Entactogens Effect: Stimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-N-methylbutan-2-amine Formula: $C_{12}H_{17}NO_2$ Mw: 207,27 g/mol	-----

Table S2.1
Information on drugs analyzed in this study.

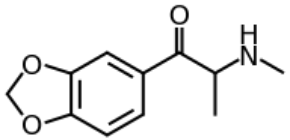
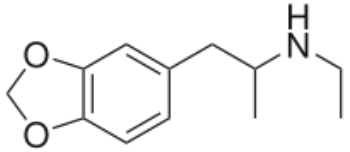
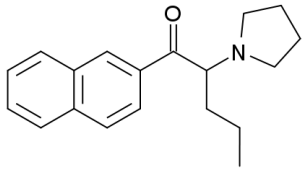
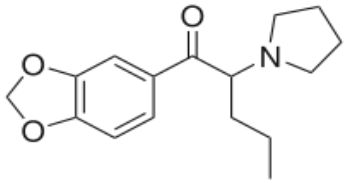
Name	Classification	Formula	Features	Deuterated
Methylone (METONE)	Family: Entactogens Effect: Stimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one Formula: $C_{11}H_{13}NO_3$ Mw: 207,23 g/mol	D3: $C_{11}H_{10}D_3NO_3$
3,4-methylenedioxyethylamphetamine (MDEA)	Family: Entactogens Effect: Stimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-N-ethylpropan-2-amine Formula: $C_{12}H_{17}NO_2$ Mw: 207,27 g/mol	D5: $C_{12}H_{12}D_5NO_2$
Naphyrone (NAPH)	Family: Other psychostimulants Effect: Psychostimulant Age: New		IUPAC Name: 1-naphthalen-2-yl-2-pyrrolidin-1-ylpentan-1-one Formula: $C_{19}H_{23}NO$ Mw: 281,39 g/mol	-----
Methylenedioxypyrovalerone (MDPV)	Family: Other psychostimulants Effect: Psychostimulant Age: New		IUPAC Name: 1-(1,3-benzodioxol-5-yl)-2-pyrrolidin-1-ylpentan-1-one Formula: $C_{16}H_{21}NO_3$ Mw: 275,34 g/mol	D8: $C_{16}H_{13}D_8NO_3$

Table S2.1
Information on drugs analyzed in this study.

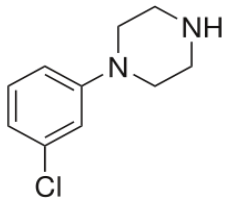
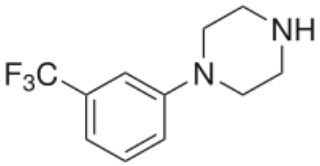
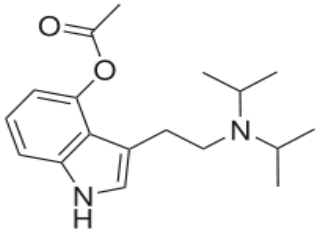
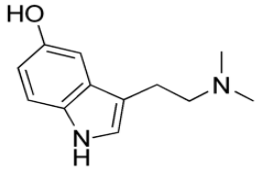
Name	Classification	Formula	Features	Deuterated
1-(3-chlorophenil)piperazine (mCPP)	Family: Piperazines Effect: Stimulant and depressant Age: New		IUPAC Name: 1-(3-Chlorophenyl)piperazine Formula: $C_{10}H_{13}ClN_2$ Mw: 196,67 g/mol	-----
1-(3-trifluoromethylphenyl)piperazine (TFMPP)	Family: Piperazines Effect: Stimulant Age: New		IUPAC Name: 1-[3-(trifluoromethyl)phenyl]piperazine Formula: $C_{11}H_{13}F_3N_2$ Mw: 230,23 g/mol	-----
4-acetoxy-N,N-dimethyltryptamine (4-AcO-DIPT)	Family: Tryptamines Effect: Hallucinogen Age: New		IUPAC Name: [3-[2-[di(propan-2-yl)amino]ethyl]-1H-indol-4-yl] acetate Formula: $C_{18}H_{26}N_2O_2$ Mw: 302,42 g/mol	-----
Bufotenine (BUF)	Family: Tryptamines Effect: Hallucinogen Age: New		IUPAC Name: 3-[2-(dimethylamino)ethyl]-1H-indol-5-ol Formula: $C_{12}H_{16}N_2O$ Mw: 204.26 g/mol	-----

Figure S2.1

Linearities and straight equations of the drugs of abuse analyzed.

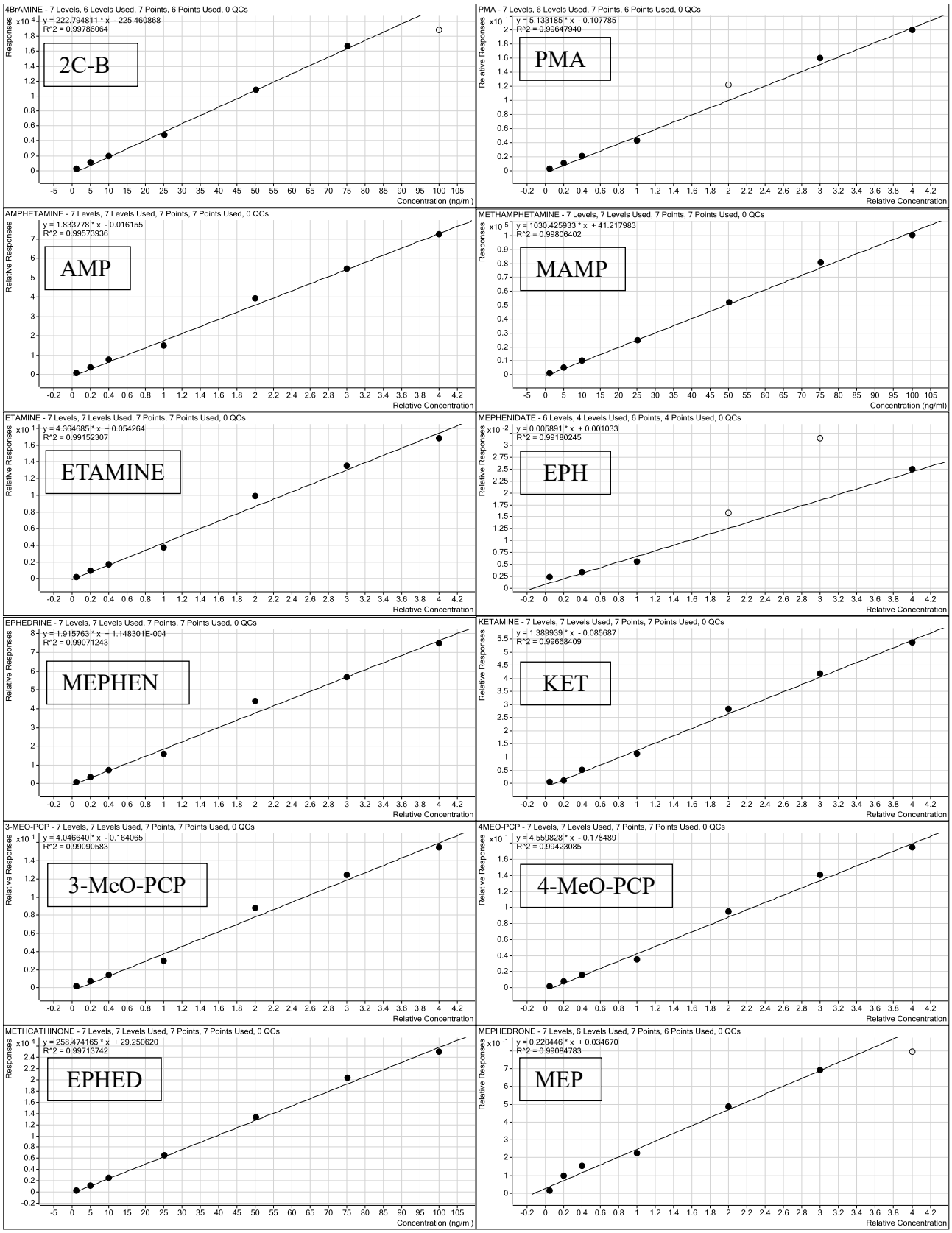


Figure S2.1

Linearities and straight equations of the drugs of abuse analyzed.

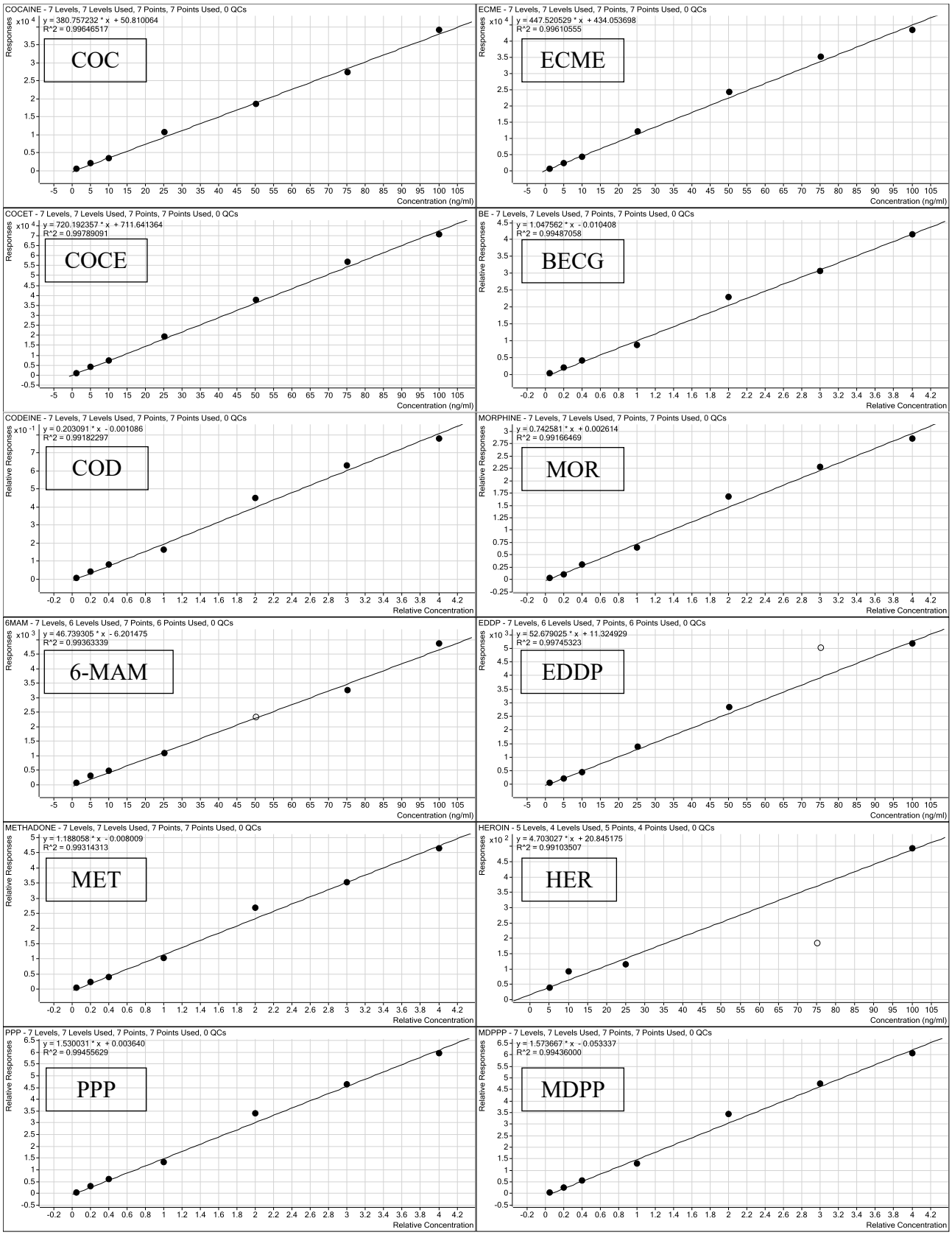


Figure S2.1

Linearities and straight equations of the drugs of abuse analyzed.

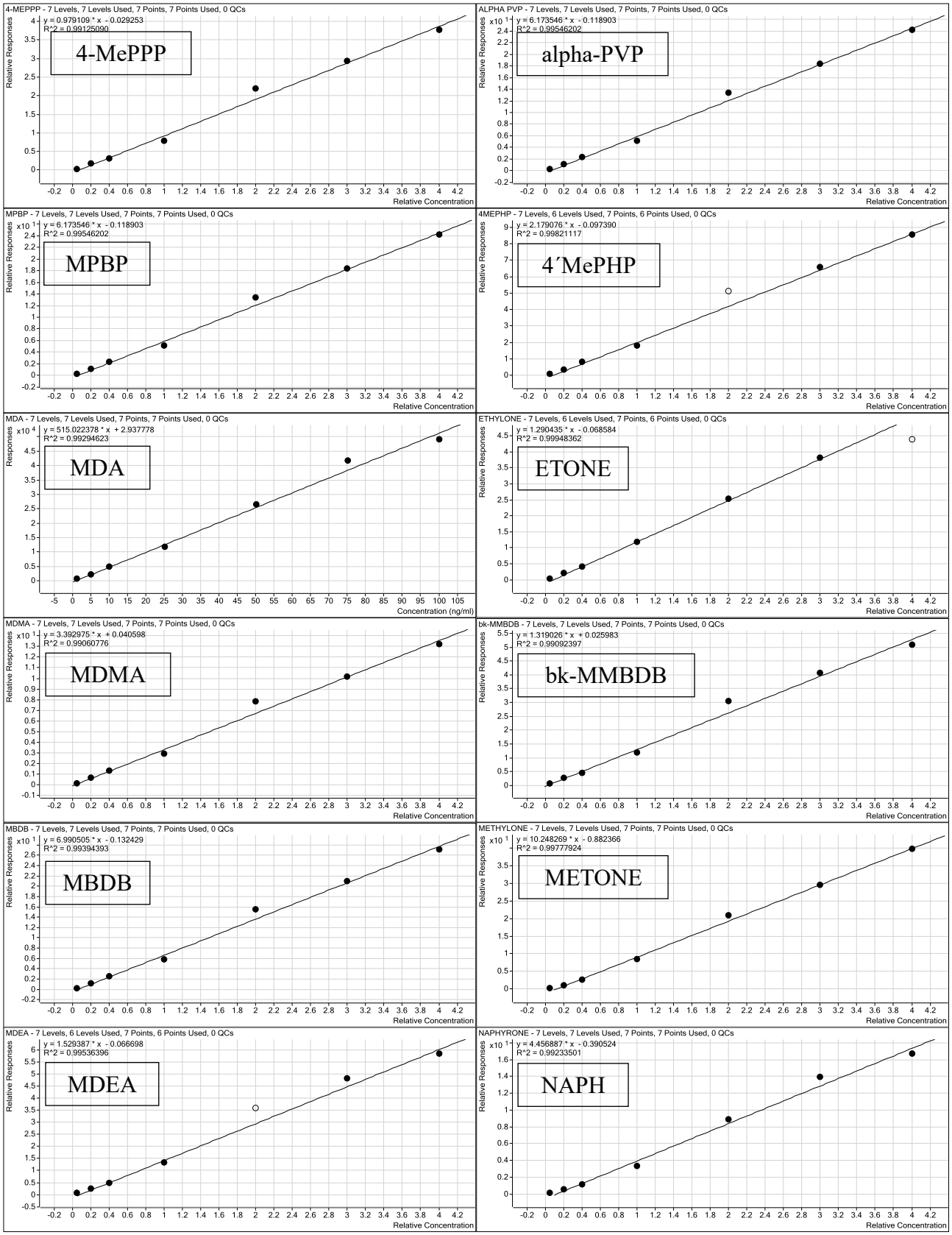


Figure S2.1

Linearities and straight equations of the drugs of abuse analyzed.

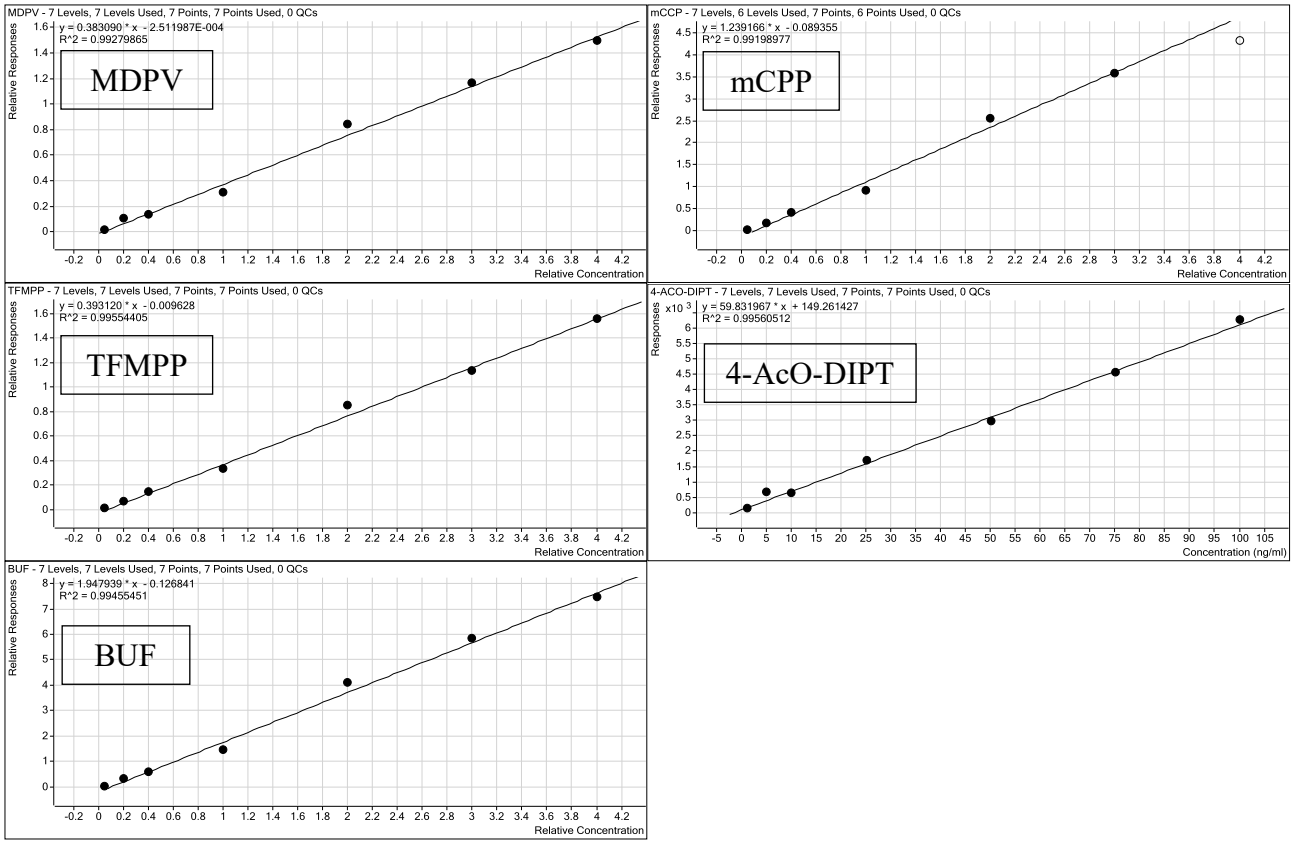


Table S2.2

Drugs obtained from the analysis of the particles of the influents of the WWTP Pinedo I. The samples were obtained between 03/04/2014 and 03/20/2014. The results are shown in ng g^{-1} (ppb). The nomenclature "EPIX" means: Pinedo I input and day of the month in wich the sample was collected, "n.d." refers to that the compound was "not detected" in the sample.

Illicit drug	EPI 4	EPI 5	EPI 6	EPI 7	EPI 8	EPI 9	EPI 10	EPI 11	EPI 12	EPI 13	EPI 14	EPI 15	EPI 16	EPI 17	EPI 18	EPI 19	EPI 20
2C-B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MAMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETAMINE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEPHEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
KET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPHED	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COC	52.86	52.53	58.31	n.d.	97.28	39.13	n.d.	27.26	37.39	36.98	20.68	126.58	n.d.	n.d.	14.24	n.d.	13.57
ECME	69.74	n.d.	n.d.	13.91	142.59	n.d.	n.d.	n.d.	31.01	n.d.	n.d.	103.49	n.d.	n.d.	113.18	66.27	n.d.
COCET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	51.29	n.d.	n.d.	n.d.	n.d.
BECG	21.48	n.d.	10.78	n.d.	137.44	107.38	n.d.	2.96	n.d.	n.d.	n.d.	76.51	56.64	n.d.	n.d.	12.88	98.23
COD	n.d.	n.d.	n.d.	n.d.	138.72	123.83	n.d.	n.d.	53.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MOR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6-MAM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EDDP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MET	77.43	159.60	173.95	21.35	121.84	119.05	52.37	86.44	52.92	70.45	97.52	145.42	189.01	n.d.	145.70	n.d.	94.69
HER	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

4-MePPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α -PVP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MPBP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4′ MePHP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
bk-MMBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
METONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDEA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NAPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPV	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mCPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TFMPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-AcO-DIPT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BUF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S2.3

Drugs obtained from the analysis of the particles of the influents of the WWTP Pinedo II. The samples were obtained between 03/04/2014 and 03/20/2014. The results are shown in ng g^{-1} (ppb). The nomenclature "EPIIX" means: Pinedo II input and day of the month in wich the sample was collected, "n.d." refers to that the compound was "not detected" in the sample.

Illicit drug	EPII4	EPII5	EPII6	EPII7	EPII8	EPII9	EPII10	EPII11	EPII12	EPII13	EPII14	EPII15	EPII16	EPII17	EPII18	EPII19	EPII20
2C-B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MAMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETAMINE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEPHEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
KET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	45.76
3-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPHED	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	36.79	30.50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	79.65
ECME	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COCET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BECG	34.92	37.55	53.62	107.84	111.42	135.65	59.09	66.39	28.96	64.09	185.31	131.18	149.00	n.d.	71.81	223.09	84.75
COD	n.d.	n.d.	80.76	254.99	318.02	n.d.	n.d.	n.d.	56.88	n.d.	n.d.	173.51	n.d.	n.d.	127.40	n.d.	105.50
MOR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6-MAM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EDDP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MET	28.02	n.d.	66.78	101.37	108.88	101.65	68.97	53.17	31.01	n.d.	141.90	82.89	117.34	n.d.	n.d.	207.34	54.87
HER	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

4-MePPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α -PVP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MPBP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4′ MePHP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
bk-MMBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
METONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDEA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NAPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPV	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mCPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TFMPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-AcO-DIPT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BUF	n.d.	n.d.	50.89	n.d.	n.d.	n.d.	63.15	n.d.	22.19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S2.4

Drugs obtained from the analysis of the particles of the influents of the WWTP Quart Benáger. The samples were obtained between 03/04/2014 and 03/20/2014. The results are shown in ng g^{-1} (ppb). The nomenclature "EQBX" means: Quart Benáger input and day of the month in wich the sample was collected, "n.d." refers to that the compound was "not detected" in the sample.

Illicit drug	EQB 5	EQB 6	EQB 7	EQB 8	EQB 9	EQB 10	EQB 11	EQB 12	EQB 13	EQB 14	EQB 15	EQB 16	EQB 17	EQB 18	EQB 19	EQB 20
2C-B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
AMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MAMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETAMINE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEPHEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
KET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPHED	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COC	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ECME	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COCET	n.d.	n.d.	n.d.	n.d.	16.49	50.31	40.23	30.69	n.d.	47.73	6.19	n.d.	n.d.	n.d.	n.d.	8.13
BECG	20.22	62.92	119.90	83.24	n.d.	n.d.	n.d.	n.d.	40.31	74.80	50.58	15.28	68.53	70.83	62.86	14.86
COD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	128.44	n.d.
MOR	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6-MAM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EDDP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HER	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

4-MePPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α -PVP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MPBP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4´ MePHP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
bk-MMBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
METONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDEA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NAPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPV	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mCPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TFMPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-AcO-DIPT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BUF	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table S2.5

Drugs obtained from the analysis of the sludges of the WWTP Pinedo I and Pinedo II. The samples were obtained between 03/04/2014 and 03/20/2014. The results are shown in ng g^{-1} (ppb). The nomenclautre "LPX" means: Pinedo sludge and day of the month in wich the sample was collected, "n.d." refers to that the compound was "not detected" in the sample.

Illicit drug	LP4	LP5	LP6	LP7	LP8	LP9	LP10	LP11	LP12	LP13	LP14	LP15	LP16	LP17	LP18	LP19	LP20
2C-B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PMA	n.d.	n.d.	n.d.	13.54	n.d.	n.d.	n.d.	n.d.	13.98	n.d.	10.86	10.55	n.d.	n.d.	8.27	15.82	16.21
AMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MAMP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETAMINE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEPHEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
KET	n.d.	n.d.	1.77	n.d.	1.77	2.04	n.d.	n.d.	1.81	n.d.	n.d.	n.d.	n.d.	2.02	n.d.	n.d.	n.d.
3-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.44	n.d.
EPHED	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MEP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COC	13.36	22.04	21.49	17.85	n.d.	31.14	4.49	n.d.	n.d.	18.97	n.d.	n.d.	31.13	58.23	12.31	36.49	41.73
ECME	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.67	n.d.	n.d.	8.87
COCET	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.27	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BECG	1.50	1.73	1.58	1.69	1.61	1.48	1.81	1.85	1.66	1.35	1.61	1.33	1.70	2.06	3.79	1.82	1.67
COD	16.48	11.06	12.48	17.23	7.81	8.54	12.84	18.18	18.56	20.55	15.55	22.46	24.34	17.44	15.76	34.16	27.16
MOR	41.14	52.30	60.22	60.48	41.95	84.09	62.79	47.23	56.60	44.69	23.69	32.05	44.05	64.24	39.62	60.81	59.67
6-MAM	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EDDP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MET	11.97	15.94	16.82	20.39	15.00	21.16	14.97	17.94	21.48	13.73	10.81	13.21	13.17	17.55	5.17	14.92	15.04
HER	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

4-MePPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α -PVP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MPBP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4′ MePHP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
ETONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDMA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
bk-MMBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBDB	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
METONE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDEA	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NAPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MDPV	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
mCPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TFMPP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4-AcO-DIPT	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BUF	3.19	2.87	2.75	2.44	n.d.	2.70	n.d.	2.69	n.d.	n.d.	4.83	n.d.	n.d.	3.03	n.d.	n.d.	n.d.

Table S2.6

Drugs obtained from the analysis of the sludges of the WWTP Quart Benáger. The samples were obtained between 03/04/2014 and 03/20/2014. The results are shown in ng g^{-1} (ppb). The nomenclautre "LQBX" means: Quart Benáger sludge and day of the month in wich the sample was collected, "n.d." refers to that the compound was "not detected" in the sample, "w.s." means "without sample", of that day.

DROGA	LQB4	LQB5	LQB6	LQB7	LQB8	LQB9	LQB10	LQB11	LQB12	LQB13	LQB14	LQB15	LQB16	LQB17	LQB18	LQB19	LQB20
2C-B	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
PMA	w.s.	n.d.	26.81	39.86	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
AMP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MAMP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
ETAMINE	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MEPHEN	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
EPH	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
KET	w.s.	2.28	2.79	n.d.	w.s.	3.14	w.s.	2.65	2.11	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	2.50	n.d.
3-MeO-PCP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
4-MeO-PCP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
EPHED	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MEP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
COC	w.s.	32.06	23.79	n.d.	w.s.	15.21	w.s.	n.d.	n.d.	10.54	n.d.	w.s.	35.14	34.65	w.s.	n.d.	n.d.
ECME	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
COCET	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
BECG	w.s.	2.46	2.17	1.60	w.s.	2.56	w.s.	2.74	2.57	1.77	2.25	w.s.	2.28	1.96	w.s.	1.63	1.49
COD	w.s.	23.33	12.73	29.62	w.s.	37.88	w.s.	78.25	36.07	49.47	20.21	w.s.	24.18	15.93	w.s.	n.d.	15.11
MOR	w.s.	118.69	77.83	82.72	w.s.	98.83	w.s.	97.63	90.31	96.33	84.23	w.s.	170.56	104.18	w.s.	122.53	77.48
6-MAM	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
EDDP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MET	w.s.	5.24	5.75	5.61	w.s.	5.03	w.s.	6.90	3.01	5.14	4.18	w.s.	3.08	3.79	w.s.	3.74	1.63
HER	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
PPP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MDPPP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.

4-MePPP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
α -PVP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MPBP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
4′ MePHP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MDA	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
ETONE	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MDMA	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
bk-MMBDB	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MBDB	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
METONE	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MDEA	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
NAPH	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
MDPV	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
mCPP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
TFMPP	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
4-AcO-DIPT	w.s.	n.d.	n.d.	n.d.	w.s.	n.d.	w.s.	n.d.	n.d.	n.d.	n.d.	w.s.	n.d.	n.d.	w.s.	n.d.	n.d.
BUF	w.s.	4.18	2.66	41.63	w.s.	11.92	w.s.	23.87	24.70	32.73	10.07	w.s.	9.25	9.92	w.s.	17.72	2.19

Table S2.7

Drugs obtained from the analysis of the Turia river sediments. The results are shown in ng g^{-1} (ppb). La nomenclatura "ESEDX" means: Sediment and the reference number of the sample, "n.d." refers to that the compound was "not detected" in the sample.

Illicit drug	ESED14	ESED17	ESED202	ESED204	ESED214
2C-B	n.d.	n.d.	n.d.	n.d.	n.d.
PMA	n.d.	n.d.	n.d.	n.d.	n.d.
AMP	n.d.	n.d.	n.d.	n.d.	n.d.
MAMP	n.d.	n.d.	n.d.	n.d.	n.d.
ETAMINE	n.d.	n.d.	n.d.	n.d.	n.d.
MEPHEN	n.d.	n.d.	n.d.	n.d.	n.d.
EPH	n.d.	n.d.	n.d.	n.d.	n.d.
KET	n.d.	n.d.	n.d.	n.d.	n.d.
3-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.
4-MeO-PCP	n.d.	n.d.	1.33	n.d.	n.d.
EPHED	n.d.	n.d.	n.d.	n.d.	n.d.
MEP	n.d.	n.d.	n.d.	n.d.	n.d.
COC	n.d.	30.02	n.d.	n.d.	n.d.
ECME	n.d.	n.d.	n.d.	n.d.	n.d.
COCET	n.d.	n.d.	n.d.	n.d.	n.d.
BECG	n.d.	0.96	n.d.	0.94	n.d.
COD	n.d.	n.d.	n.d.	n.d.	n.d.
MOR	n.d.	n.d.	n.d.	n.d.	n.d.
6-MAM	n.d.	n.d.	n.d.	n.d.	n.d.
EDDP	n.d.	n.d.	n.d.	n.d.	n.d.
MET	0.53	0.52	0.48	0.51	0.29
HER	n.d.	n.d.	n.d.	n.d.	n.d.
PPP	n.d.	n.d.	n.d.	n.d.	n.d.
MDPPP	n.d.	n.d.	n.d.	n.d.	n.d.
4-MePPP	n.d.	n.d.	n.d.	n.d.	n.d.
α -PVP	n.d.	n.d.	n.d.	n.d.	n.d.
MPBP	n.d.	n.d.	n.d.	n.d.	n.d.
4′ MePHP	n.d.	n.d.	n.d.	n.d.	n.d.
MDA	n.d.	n.d.	n.d.	n.d.	n.d.
ETONE	n.d.	n.d.	n.d.	n.d.	n.d.
MDMA	n.d.	n.d.	n.d.	n.d.	n.d.
bk-MMBDB	n.d.	n.d.	n.d.	n.d.	n.d.
MBDB	n.d.	n.d.	n.d.	n.d.	n.d.
METONE	n.d.	n.d.	n.d.	n.d.	n.d.
MDEA	n.d.	n.d.	n.d.	n.d.	n.d.
NAPH	n.d.	n.d.	n.d.	n.d.	n.d.
MDPV	n.d.	n.d.	n.d.	n.d.	n.d.
mCPP	n.d.	n.d.	n.d.	n.d.	n.d.
TFMPP	n.d.	n.d.	n.d.	n.d.	n.d.
4-AcO-DIPT	n.d.	n.d.	n.d.	n.d.	n.d.
BUF	n.d.	n.d.	n.d.	n.d.	n.d.

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Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer

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Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer

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ABSTRACT

Emerging drugs of abuse, belonging to many different chemical classes, are attracting users with promises of "legal" highs and easy access via internet. Prevalence of their consumption and abuse through wastewater-based epidemiology can only be realized if a suitable analytical screening procedure exists to detect and quantify them in water. Solid-phase extraction and ultra-high performance liquid chromatography quadrupole time-of-flight-mass spectrometry (UHPLC–QqTOF–MS/MS) was applied for rapid suspect screening as well as for the quantitative determination of 42 illicit drugs and metabolites in water. Using this platform, we were able to identify amphetamines, tryptamines, piperazines, pyrrolidinophenones, arylcyclohexylamines, cocainics, opioids and cannabinoids. Additionally, paracetamol, carbamazepine, ibersartan, valsartan, sulfamethoxazole, terbumeton, diuron, etc. (including degradation products as 3-hydroxy carbamazepine or deethylterbuthylazine) were detected. This method encompasses easy sample preparation and rapid identification of psychoactive drugs against a database that cover more than 2000 compounds that ionized in positive mode, and possibility to identify metabolites and degradation products as well as unknown compounds. The method for river water, influent and effluents samples was fully validated for the target psychoactive substances including assessment of matrix effects (-88–67.8%), recovery (42–115%), precision (<19%) and limits of quantification (1–100 ng L^{-1}). Method efficiency was thoroughly investigated for a wide range of waste and surface waters. Robust and repeatable functioning of this platform in the screening, identification and quantification of traditional and new psychoactive drugs biomarkers and other water contaminants is demonstrated.

1. Introduction

Since 2005, there is a growing interest in determining psychoactive substances and/or their metabolites in influent wastewater as an additional tool to assess their community use in a direct, quick and objective way [1–5]. There are a number of reviews covering methods for the analysis of the most frequent illicit drugs in water: methamphetamine (MAMP), amphetamine (AMP), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA), delta-9-tetrahydrocannabinol (THC), 11-hydroxy-delta-9-tetrahydrocannabinol carboxylic acid (THC-COOH), cocaine (COC), benzoylecgonine (BECG), ecgonine methyl ester (ECME), cocaethylene (COCET), heroin (HER), 6-monoacetylmorphine (6-MAM), morphine (MOR), codeine (COD) and dihydrocodeine [6–9]. Recently, the analysis of influent wastewater has acquired considerable value in comparison to other surveys, particularly, in epidemiology [10]. Advanced analytical instrumentation and methods are continuously developed to identify chemical constituents of products as well as drugs and their metabolites [11–16].

Nowadays, the pattern of these compounds changes continuously since uncontrolled recreational drugs are proliferating in number and variety. Minor modifications of the chemical structure of established drugs produce new ones not regulated by current laws and an ever-changing range of clinical effects. Many of these substances can be categorized by chemical classes in synthetic cannabinoids, synthetic cathinones, phenethylamines, piperazines, etc. . . [17,18]. The detection and reliable identifica-

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tion of these emerging drugs are challenging in several application areas, including clinical and forensic toxicology, doping-control and environmental analysis due to the similarity in their chemical structures, the lack of mass spectra in common analytical libraries, and the limited availability of reference materials [19]. The development of modern and powerful analytical methods is required for the rapid detection of the large number of design drugs that can be found in products easily obtainable by Internet or smart shops. Recently, Ibañez et al. [20] reviewed several analytical strategies that could be of application to identify different types of emerging drugs in several products simultaneously (i.e., herbal blends, powders, pills and drinkable solutions). Since then, psychoactive cathinones and tryptamines from aqueous phase samples were evaluated by positive ion monitoring mode with an atmospheric pressure ion mobility time-of-flight mass spectrometer (APIM(TOF)-MS) [21]. Diphenyl-2-pyrrolidinemethanol (D2PM) was identified in rat urine using gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-high resolution-tandem mass spectrometry (LC–HR–MS/MS) [22]. Urinary metabolites of synthetic cannabinoids, as well as the parent compounds, were also qualitatively recognized by nominal LC–MS/MS against a database [23]. Few attempts to analyze some of these compounds in wastewater using target LC–MS/MS has been performed. Lai et al. [24] determined benzylpiperazine, mephedrone (MEP) and methylone (METONE). Chen et al. [25] targeted MDMA and some of the most reported synthetic cathinones and piperazines using solid-phase extraction (SPE). Van Nujis et al. [26] extended an already reported method to determine the cathinone derivatives methylenedioxypyrovalerone (MDPV) and MEP. Very few studies analyzed a number of new psychoactive substances in wastewater or particulate matter, sewage sludge and sediment [27–32]. Nevertheless, these studies have thus far been limited in scope by either considering a narrow panel of emerging drugs of abuse or failing to examine possible metabolites formation in human body or wastewaters.

In the present study, a generic SPE LC–MS method using ultra high performance liquid chromatography coupled to quadrupole time-of-flight (UHPLC-QqTOF) was developed for the quantification of 42 illicit drugs in river and wastewaters. The selected analytes include 21 emerging psychoactive compounds (amphetamines, arylcyclohexylamines, cannabinoids, piperazines, pyrrolidinophenones, tryptamines) as well as 15 traditional ones (cocainics, opioids, cannabinoids, amphetamines) and 6 of their major urinary metabolites (Supporting information (SI-A), Table S3.1). To our knowledge, neither the simultaneous determination of these compounds nor the water analysis of many of them were previously reported. The applicability of the proposed method was proved in both, river and wastewaters. Also, different approaches for the post-run search of additional compounds and metabolites in the recorded UHPLC-QqTOF-MS/MS chromatograms are discussed, with special attention focused on the metabolites of the emerging drugs of abuse. This methodology offers advantages in comparison with previously reported methods for its versatility due to the abil-ity to acquire simultaneously qualitative information of the sample and quantitative data of the selected compounds.

2. Experimental section

2.1. Reagents and materials

High purity (>99%) standard solutions of AMP, MAMP, ethylamphetamine (ETAMINE), ephedrine (EPH), ephedrone (EPHED), methylphenidate (MEPHEN), MEP, METONE, MDMA, MDA, MDEA, N-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB), dibutylone (bk-MMBDB), 4-Bromo-2,5-dimethoxyphenethylamine (2C-B), naphyrone (NAPH), 3,4-methylenedioxypyrovalerone (MDPV), *p*-methoxyamphetamine (PMA), 4-acetoxy-*N*,*N*-dimethyltryptamine (4-AcO-DIPT), bufotenine (BUF), 1-(3-chlorophenil)piperazine (mCPP), 1-(3-trifluoromethylphenyl)piperazine (TFMPP), α-pyrrolidinopropiophenone (PPP), α-pyrrolidinovalerophenone (α-PVP), 3′,4′-methylenedioxy-α-pyrrolidinopropiophenone (MDPPP), 4-methyl- α-pyrrolidinopropiophenone (4-MePPP), 4′-methyl-α-pyrrolidinohexanophenone (4′MePHP), 4-methyl-α-pyrrolidinobutirophenone (MPBP), 4-methoxy phencyclidine (4-MeO-PCP), ketamine (KET), COC, BECG, COCET, ECME, 6-MAM, COD, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), HER, methadone (MET), MOR, JWH-018, THC, at 1000 mg L^{-1} and THC-COOH at 100 mg L^{-1} in methanol or acetonitrile were obtained from Cerillant (Austin, TX, USA) and LGC GMBH (Luckenwalde, Germany). AMP-d5, MAMP-d5, METONE-d3, MDMA-d5, MDA-d5, MDEA-d5, KET-d4, COC-d3, BECG-d3, COCET-d3, ECME-d3, 6-MAM-d3, EDDP-d3, HER-d9, MET-d3, MOR-d3, THC-d3 and THC-COOH-d3 at a concentration of 100 mg L^{-1} in methanol were also from Cerillant (Austin, TX, USA) and LGC GMBH (Luckenwalde, Germany). Deuterated compounds were used as internal standards (IS). Working standard solutions were prepared at different concentrations by appropriate dilution of the individual stock solutions in methanol-water (1:9, v/v). Calibration standards were prepared by serial dilution of the mixed working solution. Stock and working solutions were stored at −20 °C in the dark.

Water used for preparation of calibration standards and LC–MS mobile phase was purified by an Elix Milli-Q system (Millipore, Billerica, MA, USA). Methanol was purchased from Panreac (Castellar del Vallès, Barcelona, Spain) and formic acid (≥95%) was purchased from Amresco (Solon, OH, USA).

2.2. Sampling

The developed method was applied to determine the concentrations of psychoactive compounds in 21 influent and 21 effluent samples collected from 3 WWTPs and 25 surface waters from Turia River. The 24-h composite sampling was used for wastewater and grab sampling for river water. All samples were stored in polyethylene terephthalate (PET) bottles and once arrived at the laboratory were immediately frozen at −20 °C until analysis to prevent degradation of the psychoactive compounds.

2.3. Extraction procedure

Samples (250 mL) were vacuum filtered with 0.45 μm retention capacity to remove solid particles before the SPE procedure. ISs were added before the filtering step to 250 mL of water samples to obtain a final concentration of 25 μg L^{-1} in the extract (that means a concentration in water samples of 100 ng L^{-1}). Psychoactive substances were extracted by SPE using Phenomenex Strata-X cartridges (Torrance, Ca, USA). Conditioning of the cartridges was carried out with 6 mL methanol and 6 mL of Milli-Q water. Samples were trapped through the cartridges under vacuum at a flow rate of 10 mL min^{-1}. After that, the cartridges were washed with 6 mL of Milli-Q water and dried under vacuum for 15 min. Analytes were eluted with 6 mL of methanol followed by 3 mL of dichloromethane, evaporated to dryness and dissolved in 1 mL of water-methanol (9:1, v/v).

2.4. UHPLC-QqTOF-MS/MS

The chromatography was performed with an Agilent 1260 Infinity (Agilent, Waldbronn, Germany), using a column Kinetex™ 1.7 μm XB-C18100A with a length of 50 × 2.1 mm manufactured by Phenomenex and maintained at temperature of 30 °C. A constant

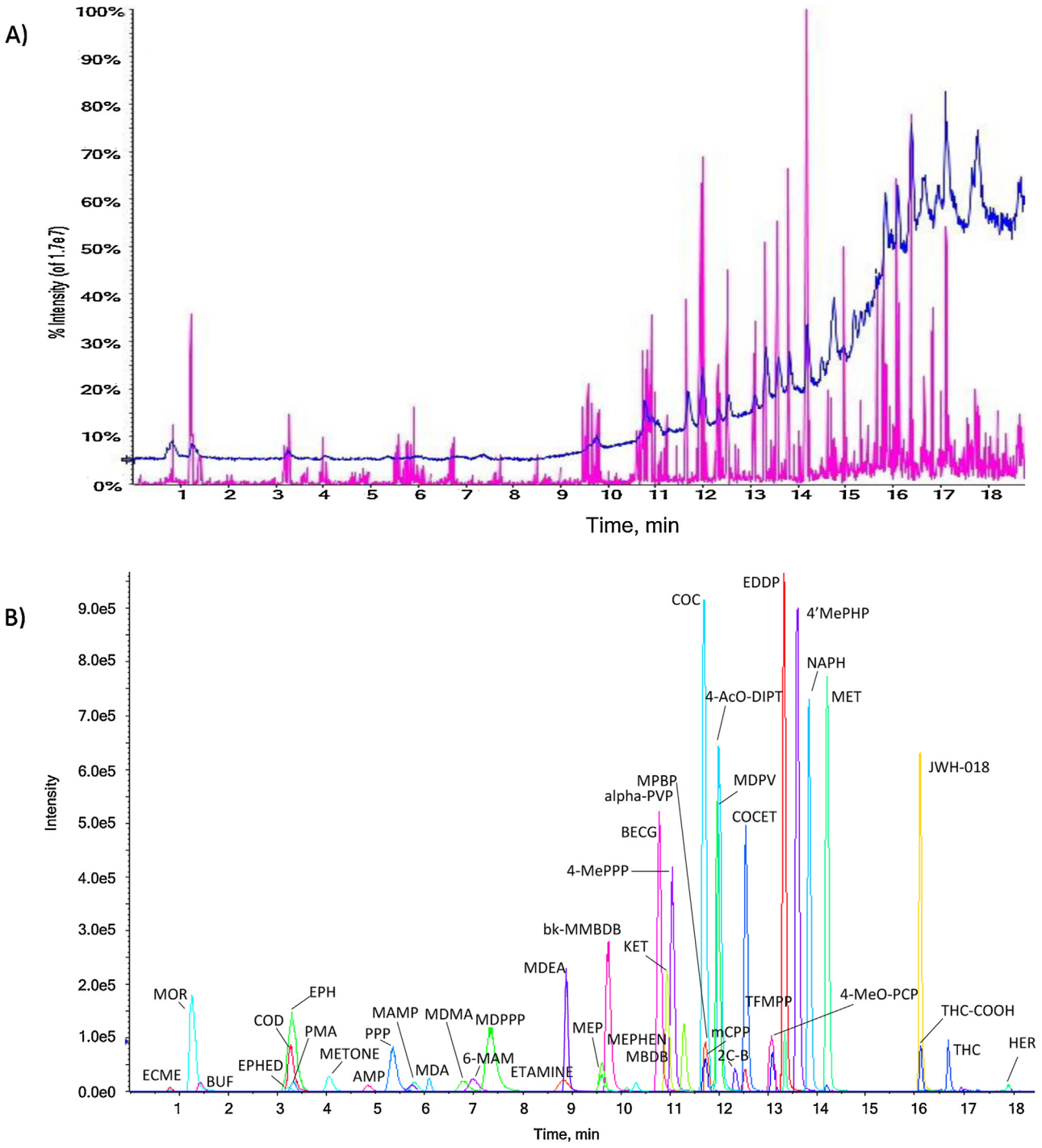


Fig. 3.1. UHPLC–QqTOF–MS/IDAMS chromatogram obtained from a spiked distilled water sample extract at 100 ng L^{-1}; A) total ion chromatogram (TIC) displaying the survey MS scan in blue and the IDA product ion scan in pink and B) extracted ion chromatogram of 42 target illicit drugs (see abbreviations in the text) (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

flow rate of 0.2 mL min^{-1} was used. The mobile phase consists of two solvents —Solvent A water with 0.1% formic acid and solvent B methanol with 0.1% formic acid. The gradient elution started at 10% B for 5 min, then increased linearly to 95% B until 12 min and continue at 95% B up to 25 min. Re-equilibration time was 15 min. The sample volume injected was 5 μL. The UHPLC system was coupled to an hybrid QqTOF ABSciex Triple TOF™ 5600 (Framingham, MA, USA). The QqTOF was calibrated as recommended by the manufacturer in MS and MS/MS in high sensitive mode. The resolution of the instrument ranged from 25,000 FWHM at *m/z* 100 (low mass) up to 40,000 at *m/z* 950 (high mass), including 34,933 FWHM at *m/z* 357.21079, at 100 spectra/s. The MS acquisition was performed using positive ionization (PI) and scan mass spectra between *m/z* 100–700 with the Turbo Ionspray source. The MS parameters were: ion spray voltage, 5000 V; declustering potential (DP), 120 V; collision energy (CE), 10; temperature 400 °C with curtain gas (CUR) 25 (arbitrary units); ion source gas 1 (GS1) 50 and ion source gas 2 (GS2) 50. The QqTOF-MS/MS instrument was calibrated every three samples using external reference compounds. The MS/MS acquisition was also performed using information-dependent acquisition

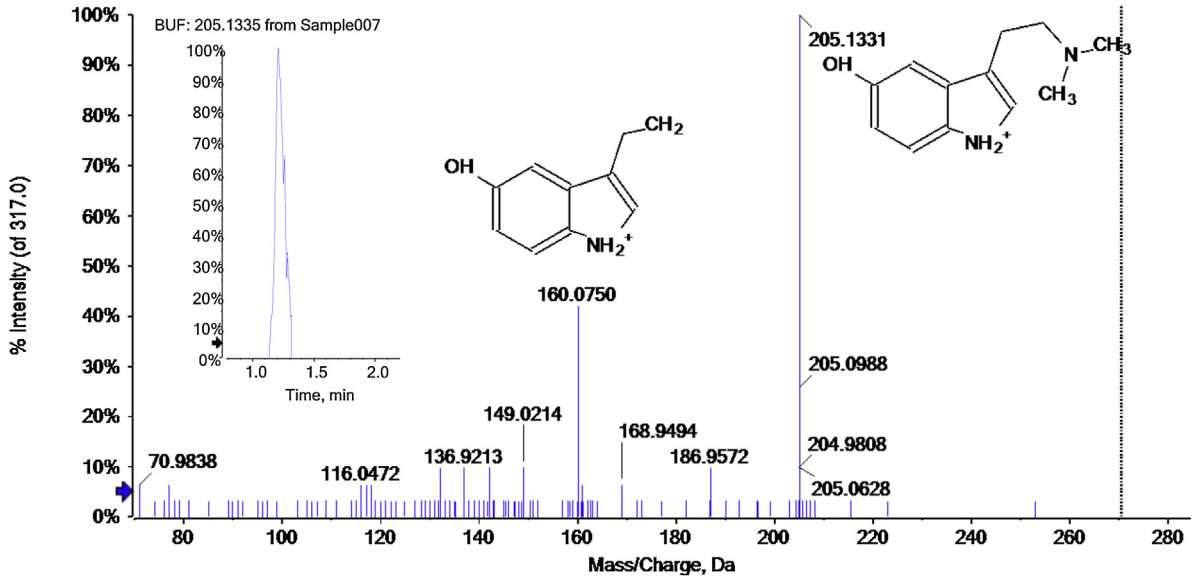


Fig. 3.2. Bufotenine MS/MS spectrum obtained in a river water sample. The insert shown the chromatographic peak extracted with a window of 0.02 mDa.

(IDA) following operating parameters: Declustering potential two (DP2), 110 V; ion release delay (IRD), 67 V; ion release width (IRW), 25 V; IDA MS/MS was performed at a fixed CE of 40 V (as a compromise to provide MS/MS mass spectra of psychoactive substances) of all ions that exceeded 1000 cps and ion tolerance of 10 mDa (isotopes higher than 4 Da were excluded). This was the selected way to analyze samples. However, in difficult cases, the IDA MS/MS of the precursor ion can be obtained at different CEs both, in a separate injection (low intensity peaks) or by adding several IDA MS/MS functions, each one at a different CE (high intensity peaks) in order to confirm compound identity.

Data acquisition and processing were carried out using software Analyst (Framingham, MA, USA), Peak View 1.0 with the application XIC Manager, MultiQuant 2.0 and MetabolitePilot 2.0 alpha software.

2.5. Validation

The lowest calibration level (LCL) tested was the concentration of each target analyte that gives an intensity $\geq 1.0 \times 10^4$ counts, as recommended elsewhere [33]. This was used to ensure that IDA MS/MS of the analyte is obtained in order to fulfil identity confirmation. Linearity was proven by preparing seven point calibration curves in water-methanol (9:1, v/v) and/or in extracts of several matrixes (influent, effluent and surface waters) within the range of LCL–1000 ng L^{-1} for each compound. Ion suppression or enhancement were evaluated by comparing the slope of the calibration curves obtained for spiked influent, effluent or surface water extracts (Sextract) with the slope of that obtained for standard prepared in water-methanol (9:1, v/v) (Sstandard) spiked at the same level (Eq. (3.1)) [34].

$$\%\text{Matrixeffect} = \left(\frac{\text{Sextract}}{\text{Sstandard}} - 1\right) \times 100 \quad (3.1)$$

Recoveries and relative standard deviations (RSDs) of selected illicit drugs were calculated in samples spiked at 100 ng L^{-1} and tested in quintuplicated. Non-spiked blank samples were always used to correct the error due to the presence of psychoactive substances in the samples by subtracting the peak areas corresponding to native analytes in the sample.

The precision of the method was determined by repeatability and reproducibility studies, expressed as the RSD (%). The intra-day precision was measured by comparing the RSD of the recovery percentages of the spiked samples carried out during the same day. The inter-day precision was determined by analyzing the spiked samples in five distinct days.

3. Results and discussion

3.1. UHPLC–QqTOF–MS/MS separation and fragmentation profiles

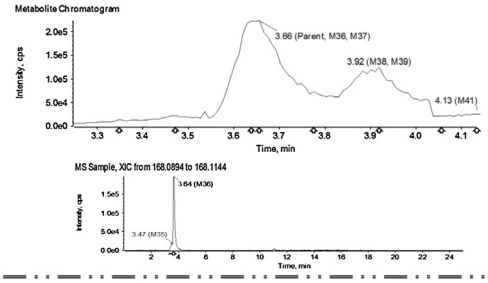
The total run time of 24 min, with analytes eluting between 0.8 and 18 min, provided efficient analysis time considering the number of illicit drugs determined and the low flow-rates compatibles with MS. Fig. 3.1 illustrates the total ion chromatogram (TIC) of both, the survey scan and the data dependent acquisition, obtained for a Milli-Q water sample spiked with the target drugs of abuse (A), and the extracted ion chromatogram (XIC) of 42 target compounds against a XIC Manager homemade table (B). Average mass accu-racy for all target drugs of abuse was below 5 ppm and the SD of these errors was from 0.1 to 0.9, which indicates a very low devi-ation of the instrument response. For the parent compounds the%of difference in the experimental and theoretical isotope ratio was always lower than 5%. Purity score values higher than 82.4% were always obtained in MS/MS identification against the homemade library carried out with the standards. Retention times of some compounds undergo slight changes due to the continued use of the analytical column. For detailed values of average mass accuracy,% of difference in the isotopical pattern, average retention time, mass spectral errors of the $[M+H]^+$ ions of targeted new and tradi-tional psychoactive compounds and the purity score obtained for the identification of the compound by an MS/MS spectra library see SI-A Table S3.2. As an example, Fig. 3.2 shows the product ion mass spectrum corresponding to BUF identified in a river water sample (MS and MS/MS of all the analytical standards are provided in SI-B, Fig. S3.1). Fragments are identified and the chromatographic peak is shown as an insert.

3.2. SPE optimization

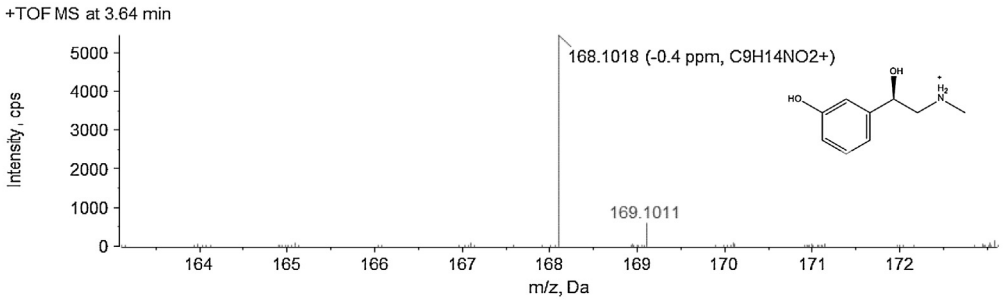
The most employed sorbents for SPE of traditional and new psychoactive compounds already tested from water samples are either hydrophilic-lipophilic balanced (HLB) reversed-phases or

A)

Peak ID	Name	Formula	m/z	ppm	R.T. (min)	Peak Area	% Area	% Score
M36	Loss of CH2 +Oxidation	C9H13NO2	168.1018	-0.4	3.64	1.85E+05	0.1	67.5



B)



C)

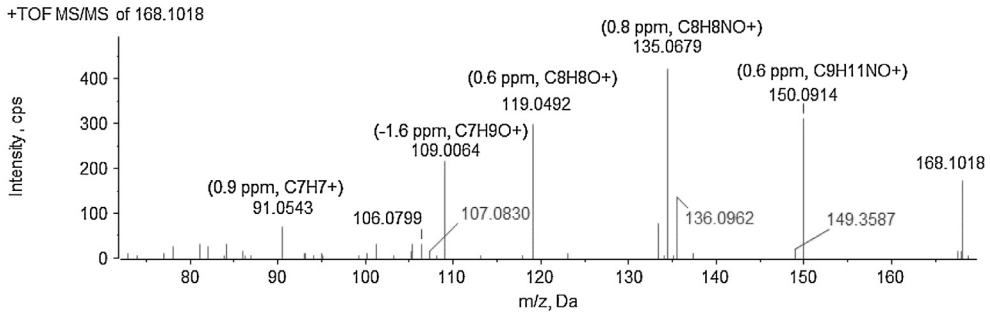


Fig. 3.3. A) Identification of an EPH metabolite B) MS and C) MS/MS formed by demethylation and oxidation obtained using the software Metabolite Pilot 2.0.

the mixed-mode (with a cation exchanger) modification of them [27,29,35]. Both provide appropriate results, but cationic exchanger required the acidification (pH < 3) being HLB more versatile. Our previous study carried out with 14 conventional illicit drugs [36] pointed out that no differences could be observed in the recoveries obtained by any of the available tradenames of HLB. Strata-X sorbent was selected for the pre-concentration of the analytes on the basis of its demonstrated performance for conventional substances. Initially, the effect of the sample pH on the retention of the analytes was investigated with 250 mL aliquots of spiked ultrapure water (100 ng L^{-1}) adjusted to pH 3, 5, 8 and 11. After loading the sample, cartridges were rinsed with 10 mL of ultrapure water adjusted to the corresponding pH and eluted with 6 mL of methanol. Recoveries at different pH demonstrated different behavior depending on the type of drugs, but most of the analytes show better recoveries at slightly basic pH of 8 (Table S3.3 in the SI-A). This pH allows to extract the 42 target analytes achiev-ing the highest recoveries for those that are poorly extracting, and acceptable ones for those that are better extracted at other pH. These results agree with those previously reported by Álvarez-Ruiz et al. [27] for a narrower pH range. Considering the slightly basic nature of the water of this area (pH 7.8–8.1) and the almost no-pH variation between samples analyzed, the recoveries remained unaffected ensuring that the analytical method is reliable, and no-pH adjustment is required to increase robustness. Recoveries were still low (up to 56%) for substances belonging to the group of cannabinoids with have logP >5. In further experiments, the elu-tion with more apolar solvents as dichloromethane was tested. The recoveries of the other analytes decrease but those of cannabinoids increase with dichloromethane. Then, in order to obtain the optimal recoveries, a sequential elution first with 6 mL of methanol to best recover all drugs, except cannabinoids, and second with 3 mL of dichloromethane to improve the recoveries of cannabinoids was carried out. Also, this combination of elution solvent is advantageous because increases the speed of the evaporation step avoiding analyte losses by volatilization. Breakthrough studies were performed, and it was found that 200 mg Strata X cartridges can concentrate up to 400 mL without significant losses for any of the investigated analytes. Working sample volumes were finally set at 250 mL to speed up the process and avoid the clogging of the sorbent bed.

3.3. Suspect screening

The suspect identification data processing was performed with Peak View 1.0 software against an XIC Manager database for positive ionization mode containing 1212 pharmaceuticals, 546 pesticides, 75 mycotoxins and other compounds, as well as the selected psychoactive substances (database listed in the SI-A as Table S3.4). In influent samples, seven compounds that belong to amphetamine group were detected, EPH was the most frequent in all analyzed samples. Two compounds of pyrrolidinophenone group —α-PVP (23.3% of the samples) and 4′MePHP (77.8%) — as well as KET (44.4% of the samples) were also found. All cocainics and opioids compounds were in most of the samples with the exception of 6-MAM and HER that were not detected even in influent

Table 3.1
Absolute recoveries (100 ng L^{-1}), matrix effects (n =5) and LCLs of the whole method for the different matrices analyzed.

Psychoactive compound	River water			Influent			Effluent			IS effect[e]
	R (%)[a]	ME (%)[b]	LCL[c] (ng L^{-1})	R (%)[a]	ME (%)[b]	LCL[d] (ng L^{-1})	R (%)[a]	ME (%)[b]	LCL[c] (ng L^{-1})	
AMPHETAMINES										
AMP	82 (12)	−5.9	15	65 (19)	−16.0	40	78 (15)	−4.0	20	C
MAMP	88 (10)	33.1	5	64 (14)	19.3	20	80 (10)	40.1	5	C
ETAMINE	84 (14)	−8.2	25	52 (18)	−13.5	50	76 (20)	−7.8	30	NC ↑
EPH	85 (12)	−2.6	1	75 (16)	−10.7	15	84 (13)	0.7	5	C
EPHED	62 (16)	−5.1	5	42 (19)	−44.5	30	61 (17)	−18.9	10	NC
MEPHEN	91 (11)	−38.2	1	80 (15)	−27.2	10	92 (11)	−22.3	2	NC↓
MEP	76 (12)	−58.2	5	65 (17)	−60.3	20	74 (13)	−64.1	10	NC↑
METONE	95 (10)	−55.4	5	75 (16)	−57.0	20	92 (12)	−44.8	10	C
MDMA	105 (10)	−53.0	10	86 (13)	−59.4	30	95 (12)	−52.5	10	C
MDA	84 (13)	−0.9	5	70 (15)	−20.7	15	83 (14)	9	10	C
MDEA	85 (12)	−3.9	5	69 (16)	−10.2	30	80 (15)	−4.5	10	C
MBDB	89 (14)	−63.6	10	72 (17)	−63.5	40	88 (15)	−61.9	10	NC↑
bk-MMBDB	90 (11)	1.9	10	86 (18)	22.2	10	92 (12)	0.2	10	NC↑
2C-B	74 (15)	−13.2	10	65 (19)	−7.5	30	72 (14)	16.0	10	NC↓
NAPH	65 (13)	−48.6	15	56 (17)	−48.4	40	63 (15)	−31.2	20	NC↓
MDPV	78 (10)	67.1	10	62 (18)	46.2	10	80 (11)	67.8	10	NC↓
PMA	94 (10)	−2.2	10	85 (12)	−7.7	20	93 (10)	0.3	10	NC↑
TRYPTAMINES										
4-AcO-DIPT	70 (14)	−86.6	50	45 (19)	−88.0	100	66 (16)	−81.2	50	NC↓
BUF	83 (13)	2.1	10	72 (20)	−13.2	40	85 (15)	−1.3	10	NC↑
PIPERAZINES										
mCPP	64 (15)	−17.3	15	54 (19)	−20.4	30	65 (16)	−9.5	15	NC↑
TFMPP	62 (14)	65.9	10	55 (18)	66.3	20	65 (16)	65.9	10	NC↓
PYRROLIDINOPHENONES										
PPP	71 (12)	−2.2	10	65 (16)	−16.1	30	68 (14)	−3.9	10	NC↑
α-PVP	74 (10)	−3.8	10	68 (17)	−14.8	30	72 (11)	−3.8	10	NC↑
MDPPP	87 (11)	−5.5	10	75 (17)	−10.0	30	85 (12)	−6.3	10	NC↑
4-MePPP	96 (10)	−62.5	20	84 (15)	3.6	20	91 (13)	12.7	20	NC↓
4′-MePHP	86 (15)	29.4	10	71 (16)	5.7	20	85 (17)	28.3	20	NC↓
MPBP	85 (12)	14.0	10	75 (14)	4.7	20	84 (13)	13.9	10	NC↑
ARYLCYCLOHEXYLAMINE										
4-MeO-PCP	75 (17)	−29.5	5	65 (11)	−32.9	20	80 (12)	−27.1	5	NC↓
KET	91 (11)	−6.0	5	82 (12)	−9.5	15	79 (9)	1.9	10	C
COCAINICS										
COC	115 (8)	−9.2	50	91 (19)	−23.8	75	94 (8)	−15	60	C
BECG	101 (12)	5.1	1	92 (20)	−10.7	10	101 (6)	3.3	5	C
COCET	82 (15)	−11.9	5	79 (18))	−21.9	15	76 (11)	−12.9	10	C
ECME	61 (19)	13.7	10	59 (15)	--	20	63 (12)	−22.5	15	C
OPIOIDS										
6- MAM	77 (9)	−6.1	20	75 (19)	−2.5	20	79 (10)	4.3	20	C
COD	75 (16)	−18.0	5	65 (21)	−31.7	15	70 (13)	−12.7	10	NC↓
EDDP	70 (13)	14.0	10	61 (19)	−11.3	30	69 (17)	−17.1	20	C
HER	78 (21)	−83.7	100	68 (20)	−87.6	100	77 (18)	−80.3	120	C
MET	75 (14)	8.3	5	65 (21)	−16.9	40	77 (9)	2.4	10	C
MOR	84 (8)	5.1	1	82 (15)	23.1	30	80 (17)	43.0	20	C
CANNABINOIDS										
JWH-018	81 (14)	0.6	15	70 (11)	−0.8	35	75 (10)	3.6	30	NC↑
THC	72 (17)	26.2	30	61 (15)	27.1	50	59 (15)	35.7	35	C
THC-COOH	78 (19)	2.3	25	66 (17)	−10.1	45	67 (19)	3.5	30	C

[a] As Average (RSD).
[b] As% of signal suppression (−) or enhancement (+) in comparison to the standards in solvent as reported in the experimental section.
[c] Calculated experimentally using samples spiked at the same level than LCLs.
[d] Estimated from samples spiked at 100 ng L^{-1}.
[e] C: corrected; NC: not corrected, ↑ and increased, ↓ but decreased.

Table 3.2
Comparison of the quantitative results obtained using the ABSciex TripleTOF™ 5600 (QqTOF) and a more traditional triple quadrupole (QQQ) for influent, effluent and river water samples.

Psychoactive compounds[a]	Sample 1 Influent (ng L^{-1})[b]		Sample 2 Effluent (ng L^{-1})[b]		Sample 3 River water (ng L^{-1})[b]	
	QqTOF	QQQ	QqTOF	QQQ	QqTOF	QQQ
AMPHETAMINES						
AMP	62 (3.4)	72 (0.4)	n.d.	n.d.	n.d.	n.d.
MAMP	8 (1.3)	7 (0.5)	n.d.	n.d.	n.d.	n.d.
ETAMINE	10 (1.0)	29 (1.4)	n.d.	n.d.	n.d.	n.d.
EPH	13 (1.2)	17 (1.2)	5 (0.4)	89 (2.4)	n.d.	n.d.
MDMA	64 (1.6)	67 (0.9)	12 (1.0)	10 (0.5)	4 (1.0)	6 (0.5)
MDA	17 (1.5)	85 (8.2)	n.d.	n.d.	n.d.	n.d.
MDEA	5 (0.5)	12 (0.4)	6 (0.3)	13 (0.4)	n.d.	n.d.
TRYPTAMINES						
BUF	n.d.	n.d.	n.d.	n.d.	65 (0.8)	5 (1.1)
ARYLCYCLOHEXYLAMINE						
KET	11 (1.1)	13 (0.7)	12 (0.9)	11 (0.2)	n.d.	n.d.
COCAININCS						
COC	550 (15.3)	618 (9.8)	n.d.	n.d.	n.d.	n.d.
BECG	1450 (125.1)	1593 (55.6)	43 (1.3)	39 (0.4)	5 (0.4)	3 (0.2)
COCET	184 (18.2)	162 (6.4)	n.d.	n.d.	n.d.	n.d.
ECME	55 (1.5)	57 (0.9)	24 (1.2)	18 (0.8)	n.d.	6 (0.4)
OPIOIDS						
COD	1527 (125.1)	969 (86.9)	482 (25.1)	744 (28.4)	7 (0.5)	2 (0.8)
EDDP	108 (4.6)	65 (6.2)	n.d.	69 (3.4)	n.d.	n.d.
MET	24 (1.4)	26 (0.9)	26 (2.3)	24 (1.5)	25 (4)	1 (0.4)
MOR	195 (4.8)	196 (3.0)	31 (2.8)	31 (1.1)	3 (1.0)	2 (0.2)
CANNABINOIDS						
THC	127 (5.6)	97 (2.4)	n.d.	n.d.	n.d.	n.d.
THC-COOH	148 (10.1)	48 (1.6)	n.d.	n.d.	n.d.	n.d.

[a] only analytes that occur in any of the samples.
[b] average (SD) $n = 3$. n.d.: non-detected.

samples. THC was detected in 55% of the samples. Regarding effluent samples, were identified almost the same compounds as in influent samples but in a fewer frequency. In river water samples, compounds as MDMA, BUF, BECG, ECME, COD, MET and MOR were sporadically identified. The frequency of detection and mean concentration of individual psychoactive compounds in the three types of samples are summarized in SI-A Table S3.5.

A total average of 165 pharmaceuticals, 7 pesticides and 25 other compounds were detected in influent samples; 45 pharmaceuticals, 7 pesticides and 3 other compounds were detected in effluent samples, and 30 pharmaceuticals, 6 pesticides and 5 other compounds in river water samples (listed in the SI-A, Table S3.6 including name, empirical formula, mass (Da), mass error (ppm), isotope ratio and purity score and illustrated the MS and MS/MS spectra of some of them in Fig. S3.2 in SI-A).

3.4. Metabolites and degradation products identification

The detection of metabolites and degradation products was carried out using the metabolite finding software (MetabolitePilot 2.0). After selection of the samples and parent compounds, the software is programmed to look for a list of possible phase I (debenzylation, deethylation, nitroreduction, demethylation, etc.) and II (hydroxylation, methylation, different conjugations, etc.) reactions and their corresponding exact mass difference expected on the product. This type of software was already successfully applied to identify metabolic pathway of conventional illicit drugs [37]. In our study, more than 25 metabolites of the traditional illicit drugs not covered as target analytes were identified, as ETAMINE and COC metabolites (an example is shown in Fig. S3.3 in SI-A). Methylefedrone was also identified in a number of samples. Fig. 3.3 shows MS and MS/MS of a potential EPH metabolite [$C_9H_{13}NO_2$] identified by the software resulting from the demethylation and oxidation and, up to our knowledge, not previously reported in wastewater. The MS/MS showed a positive match with this structure. EPH has reemerged as part of street drugs and nutritional supplements, serving both as a major source for the illicit production of methamphetamine and methcathinone, and as a mental stimulant and weight loss promoter in dietary supplements, and consequently is frequently found in wastewaters. The relationship between the occurrence of amphetaminic compounds and EPH in wastewater was proven [37]. A deeper search of different studies on EPH degradation and analogues showed that this compound matches 1-(3-hydroxyphenyl)-2-(methylamino) ethanone (HPMAE) or phenylephrine, a legal compound (analogue of EPH) used as nasal decongestant. This was confirmed by a search of the molecular formula in the METLIN database (https://metlin.scripps.edu/index.php) that shows this compound as the second match. Also, METLIN has HR-MS/MS spectra for it, which agree with that shown in Fig. 3.3. All these findings remark the good prospects of this system.

The software is helpful but it also identify a number of false metabolites. As an example, a compound with the empirical formula [$C_9H_{11}NO_2$] identified as a degradation product also resulting from the demethylation and oxidation (MS and MS/MS is in SI-A, Fig.S3.4). The MS/MS fragmentation pattern does not fit the proposed structure. Searching that formula in Chemspider (http://www.chemspider.com/), the first match would be phenylalanine, which seems to be more realistic than the EPH metabolite. A further look for phenylalanine spectra in the METLIN database demonstrated that this is actually the detected compound. This erroneous identification does not change the good prospects but illustrates the complexity of this task and the importance of revising the results in alternative sources.

3.5. Quantitative validation

The calibration curves [in water-methanol (9:1) or as a matrix matched standards] were linear with coefficients of determina-tion (R^2) ≥ 0.99 with the exception of 4-AcO-DIPT and HER (0.98) (detailed calibration equations in SI-A Table S3.7). Table 3.1 summarizes matrix effect, LCL, recovery and precision (reproducibility) for

each analyte in influent, effluent and surface waters. Matrix effect ranged from −88.0 to 66.3% for influent, from −81.2 to 67.8% for effluent and from −86.6 to 67.1% for river water.

The sensitivity in this study was not established in the orthodox way. When high resolution mass spectrometry (HRMS) is used, the concept of limits of detection and/or quantification may be ambiguous because the common definition of these parameters are based on the signal-to-noise (S/N) ratio involving its measurement. Commonly, the HRMS chromatograms are extracted against a very narrow mass window (20 mDa in this case) and in these cases the S/N is difficult to measure, due to the non-practical existence of chemical noise in the chromatogram. This was already widely discussed for the QqTOF since 2007 [38,39]. In the case of the orbitrap platforms, an arbitrary threshold of 10,000, which counts that was the minimum intensity required for a possible identification using automatic processing with their particular software, is increasingly used. Intensity threshold as well as S/N threshold can be fixed in the Xic Manager software [33]. Including only XICs with ratio S/N > 10, different intensity thresholds —100, 1000 and 10,000 counts—were studied. The intensity threshold of 100 counts identify as peaks mostly baseline noise, the threshold of 1000 counts identify small peaks that only sometimes are fragmented because the high intensity of other matrix components, in the case of a threshold of 10,000 counts the identified compounds provides always MS and confirmatory IDA MS. These results pointed out that the arbitrary threshold of 10,000 could be extended from the orbitrap to other instruments and brands, and at least, can be exported to the type/brand of QqTOF used in this study. The LCL was established between 1 and 100 ng L^{-1}. The repeatability and reproducibility were below than 16% and 19% respectively. The recoveries obtained were in the range 42 and 115%.

The quantification of the detected illicit drugs in the three matrices was performed using MultiQuant 2.0 software. The mean concentration levels detected in influent samples were COD (3689.1 ng L^{-1}) followed by BECG (2685.5 ng L^{-1}) and COC (1098.3 ng L^{-1}) and EPH (933.5 ng L^{-1}) and MDA (658.0 ng L^{-1}). In the effluent the highest concentrations were COD (1693.9 ng L^{-1}) followed by EPH (169.1 ng L^{-1}), and in river waters were COD (49.1 ng L^{-1}), MDMA (14.9 ng L^{-1}) and ECME (12.7 ng L^{-1}) (the mean value ± RSD of detected compounds is shown in SI-A Table S3.5). Table 3.2 outlines the quantification of detected illicit drugs in one influent sample compared with that obtained using UHPLC-QqQ-MS/MS (conditions detailed in SI-A Table S3.8). The results showed a good agreement between both techniques.

4. Conclusions

The UHPLC–QqTOF–MS/MS method proposed in the present study significantly advances the analysis of psychoactive substances in environmental samples. A straight forward sample preparation procedure, based on conventional SPE was extensively investigated, particularly for the new drugs of abuse; however, their generic character provides its potential to be expanded to a much broader range of contaminants, including other biomarkers currently analyzed using different analytical methods (e.g., sterols, endocrine disrupting compounds, pharmaceuticals, pesticides, flame retardants, among others). The UHPLC–QqTOF–MS based method illustrated here was able to identify several metabolites and degradation products of the new psychoactive substances selected as model compound. In addition, we identify other psychoactive substances. The key advantage of UHPLC–QqTOF–MS methods is the ability to detect species not part of classical targeted analytical procedures. The method showed appropriate sensitivity (LCLs 1 and 100 ng L^{-1}) recoveries (42–115%) and precision (<19%). The matrix effects were important although did not prevent their determination. This variety of systems are the ultimate step to achieve simultaneously qualitative and quantitative assessment of the fate, transport and impact of new substances to the environment.

Acknowledgments

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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.2016.07.062.

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Supporting Information - A

Analysis of Psychoactive Substances in Water by Information Dependent Acquisition on a Hybrid Quadrupole Time-of-Fight Mass Spectrometer

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Due to the high number of pages of *Supporting information - A*, it has been decided not to include it in this Doctoral Thesis. For any query is available at the following link: http://dx.doi.org/10.1016/j.chroma.2016.07.062

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Supporting Information - B

Analysis of Psychoactive Substances in Water by Information Dependent Acquisition on a Hybrid Quadrupole Time-of-Fight Mass Spectrometer

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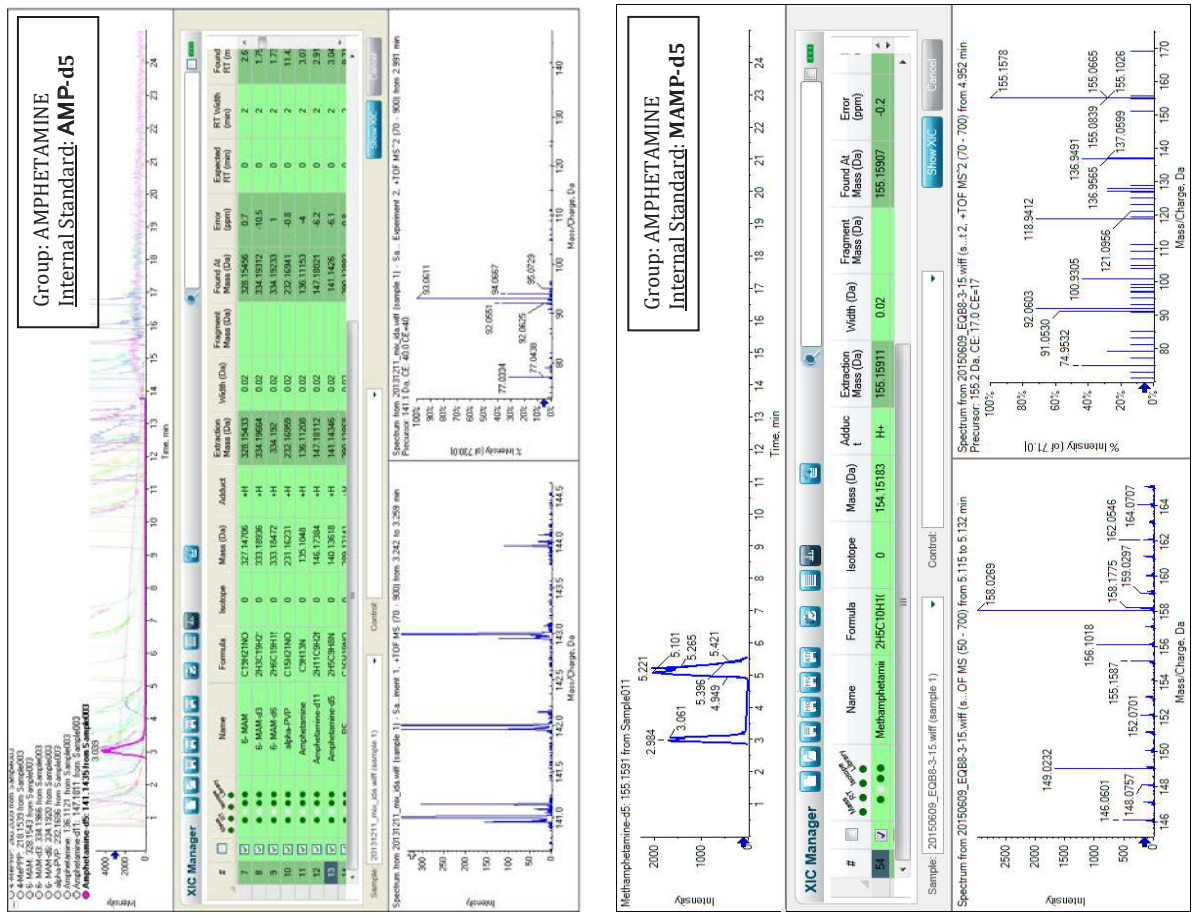
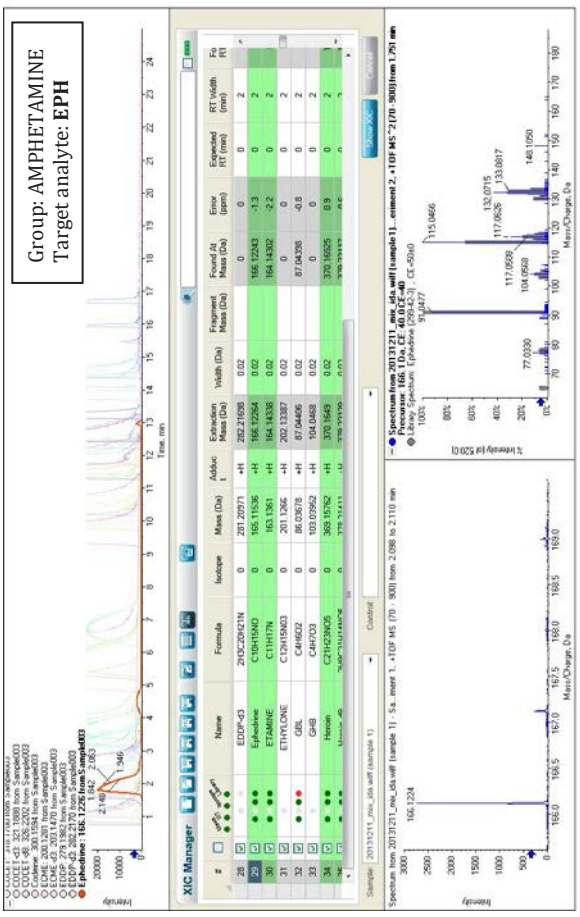


Figure S3.1. MS and MS/MS Spectra of the Target Analytes.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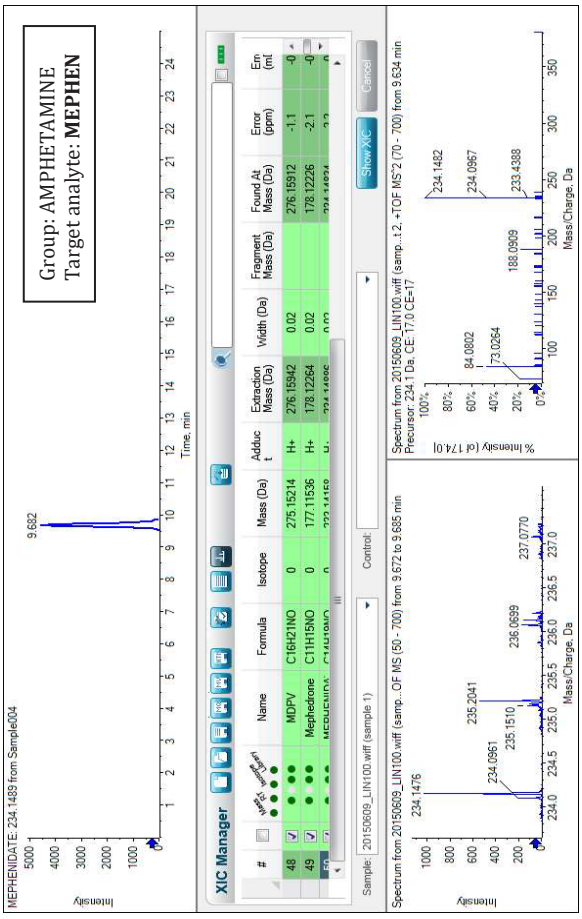
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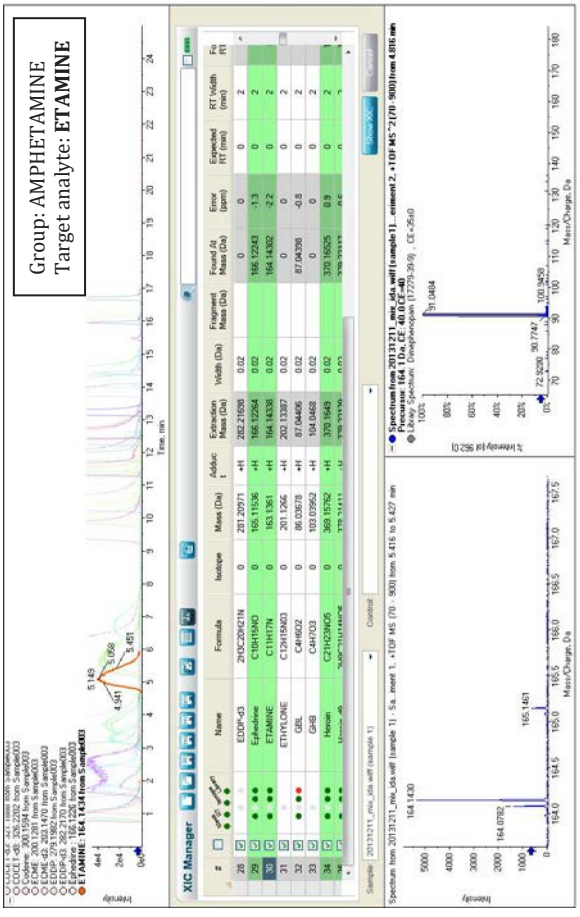
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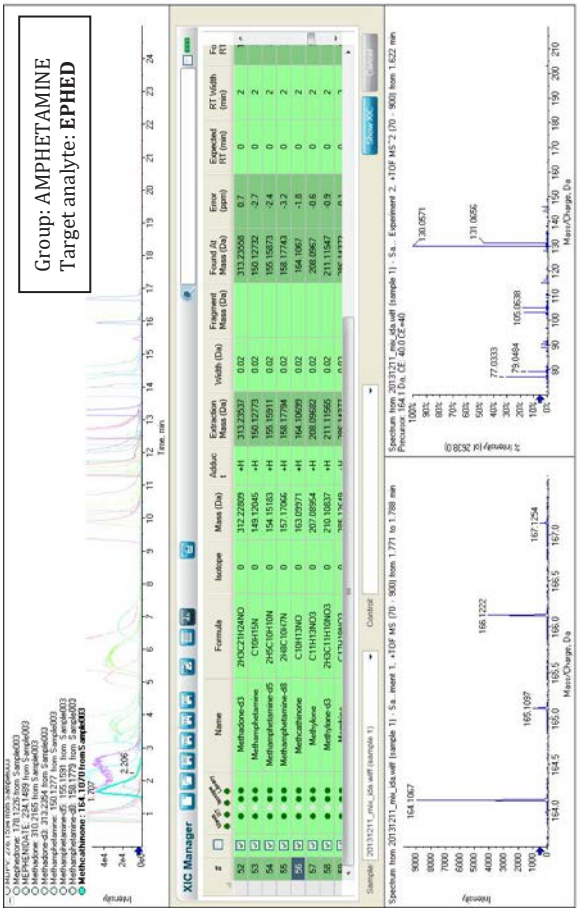
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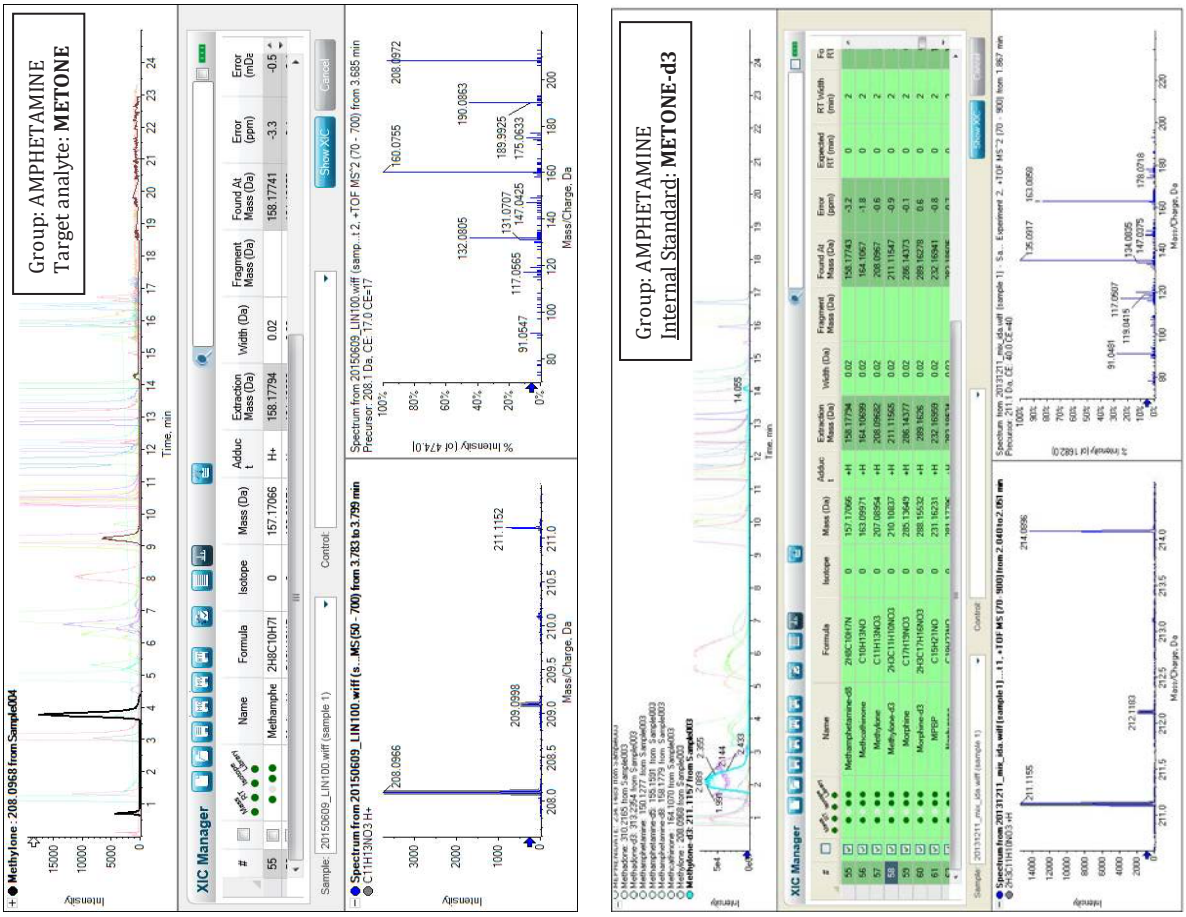
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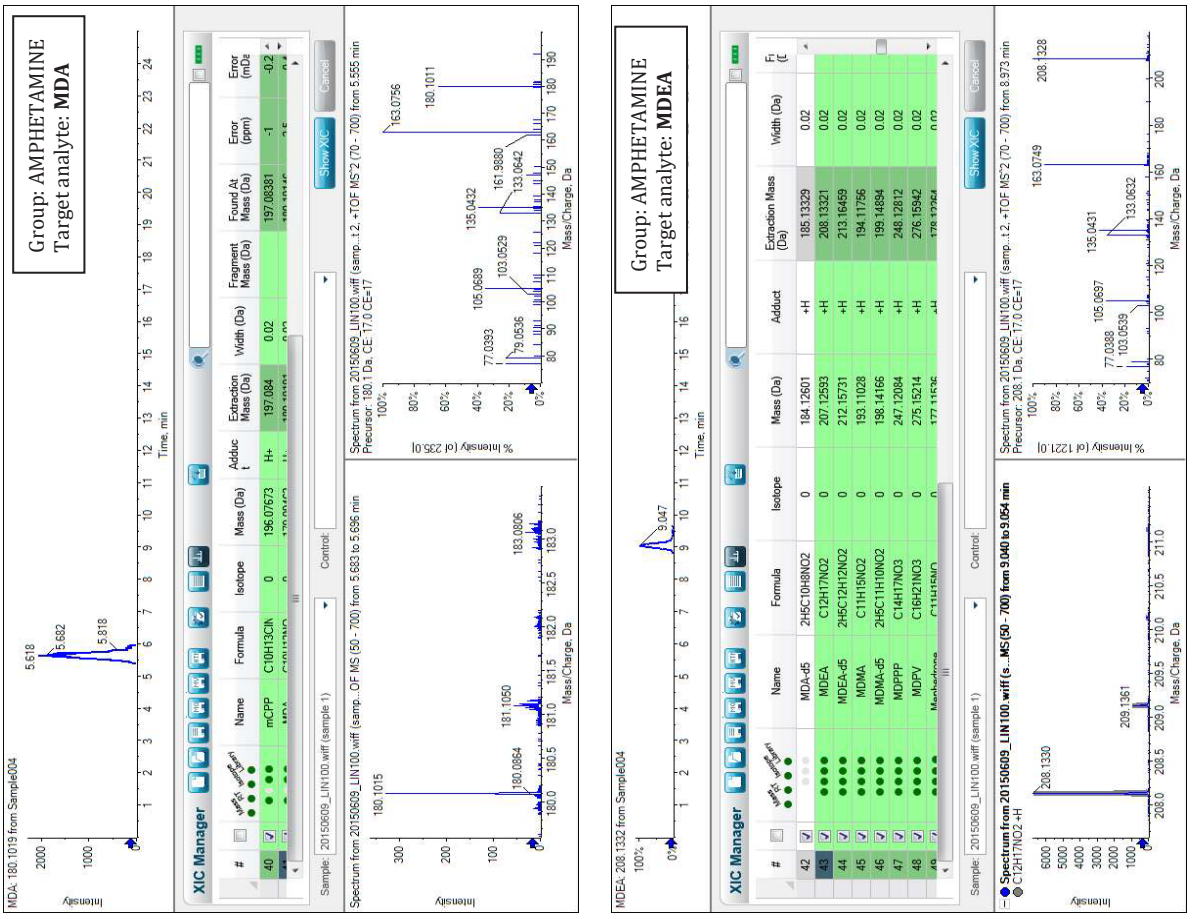
Internal Standard: METONE-d3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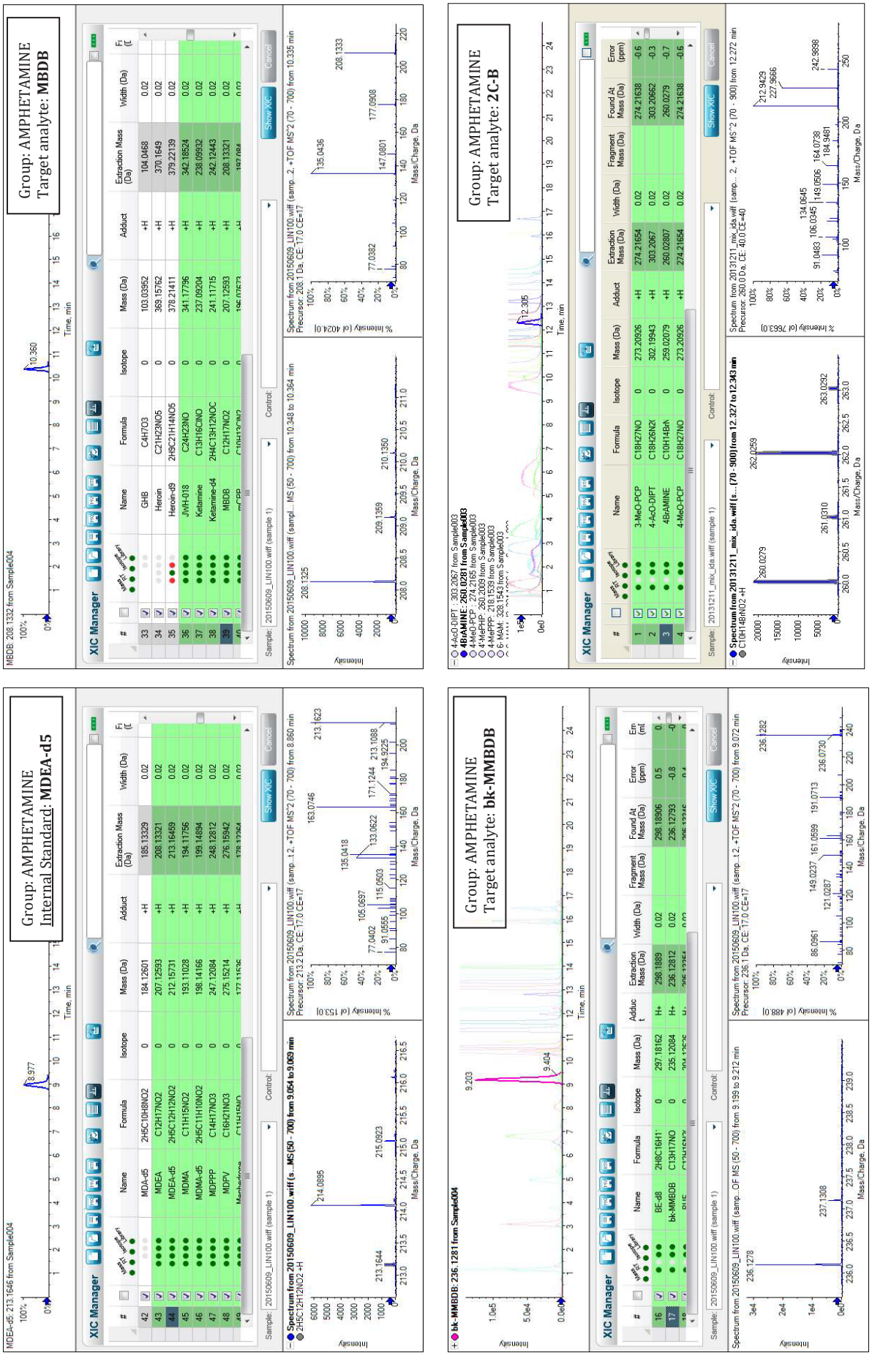
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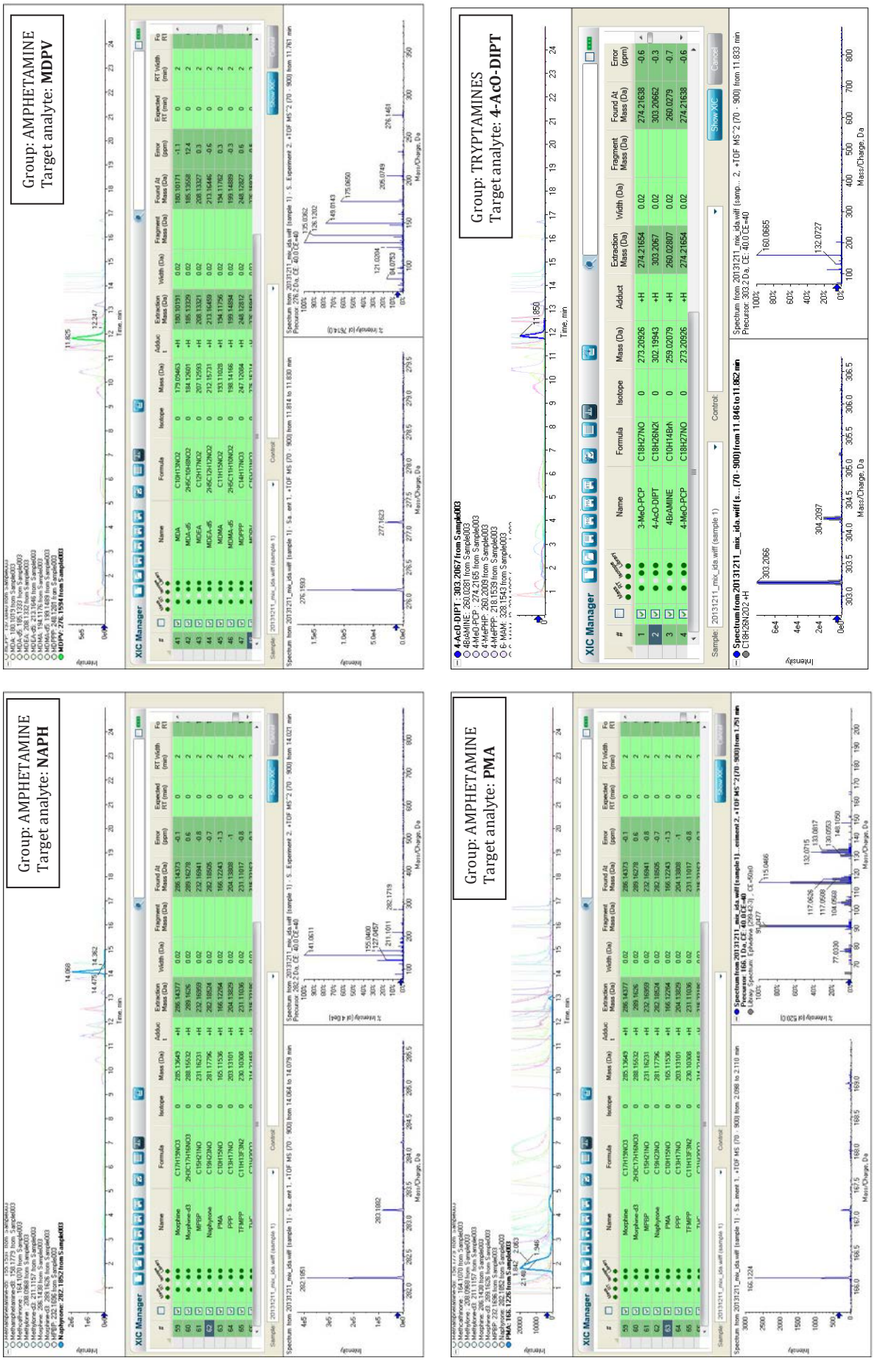
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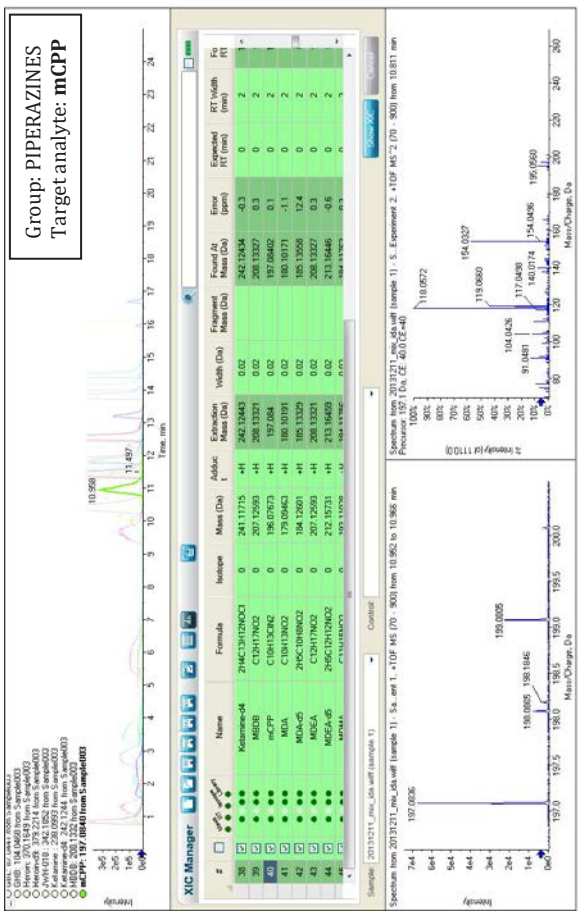
Group: TRYPTAMINES
Target analyte: 4-AcO-DIPT
Group: AMPHETAMINE
Target analyte: PMA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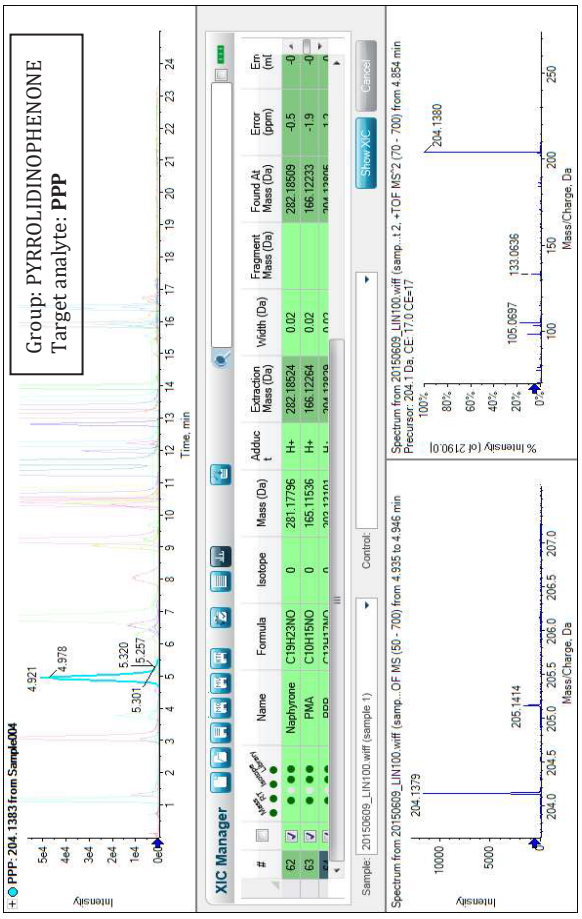
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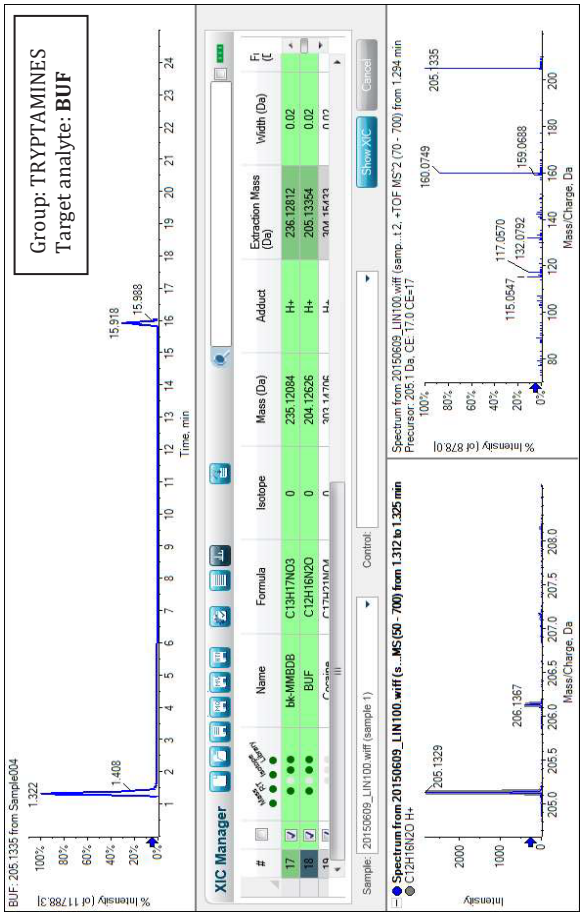
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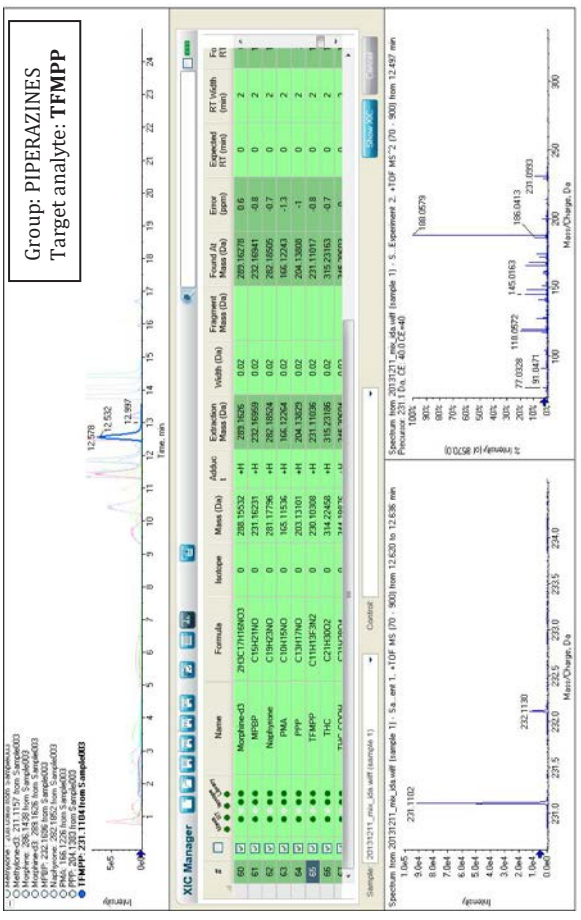
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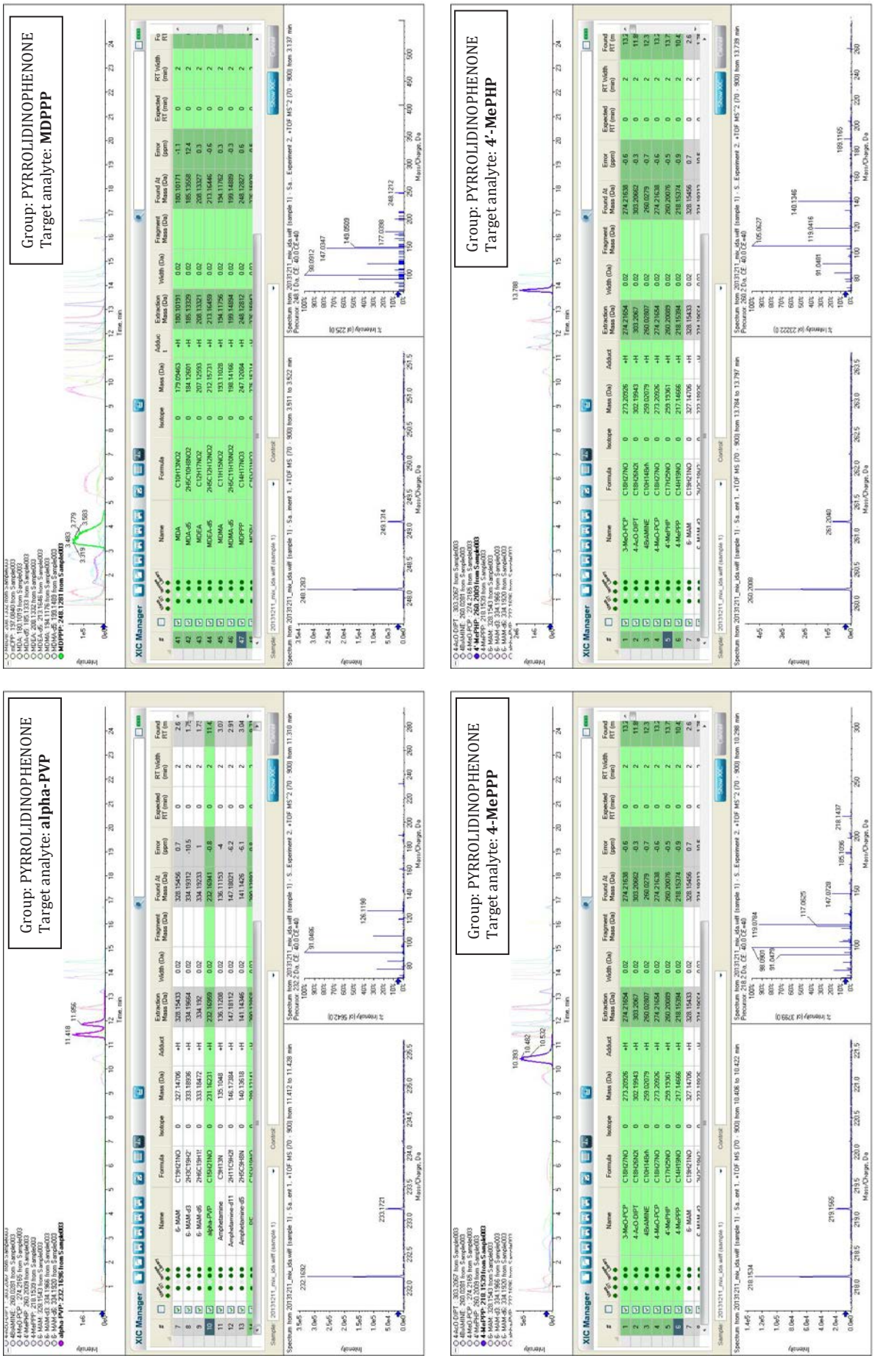
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Target analyte: 4'-MePHP
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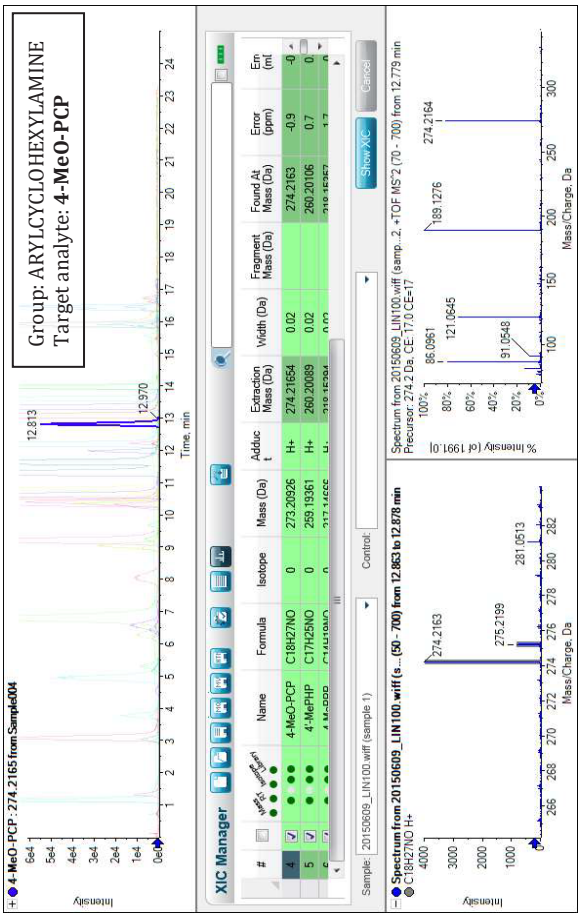
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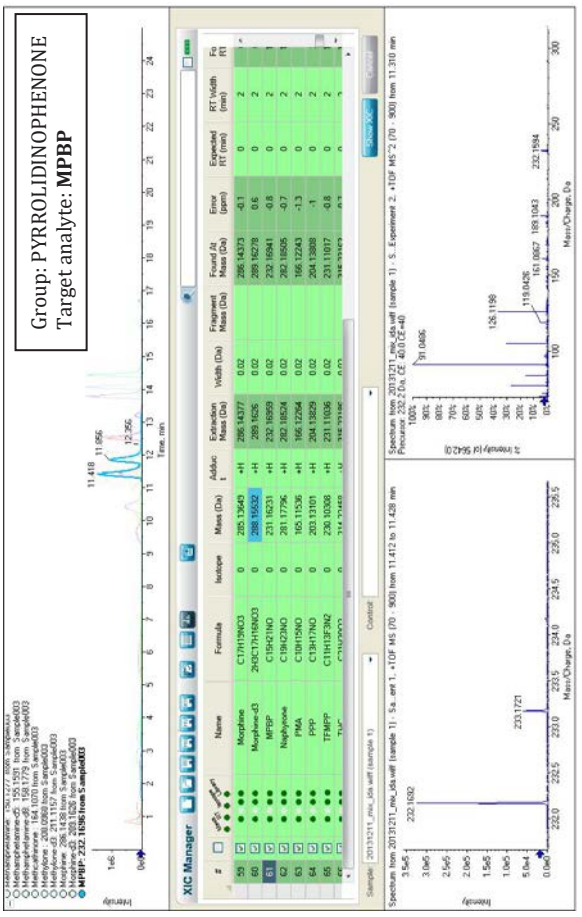
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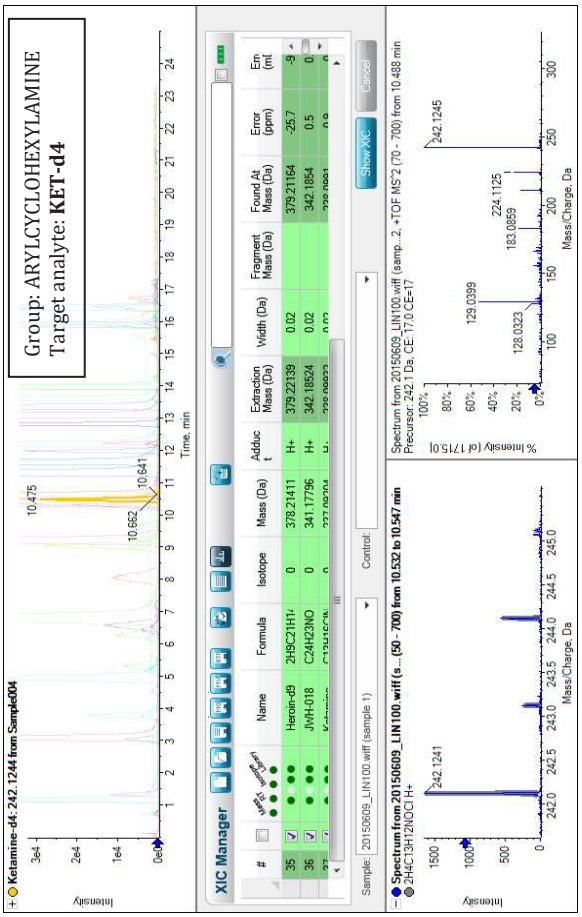
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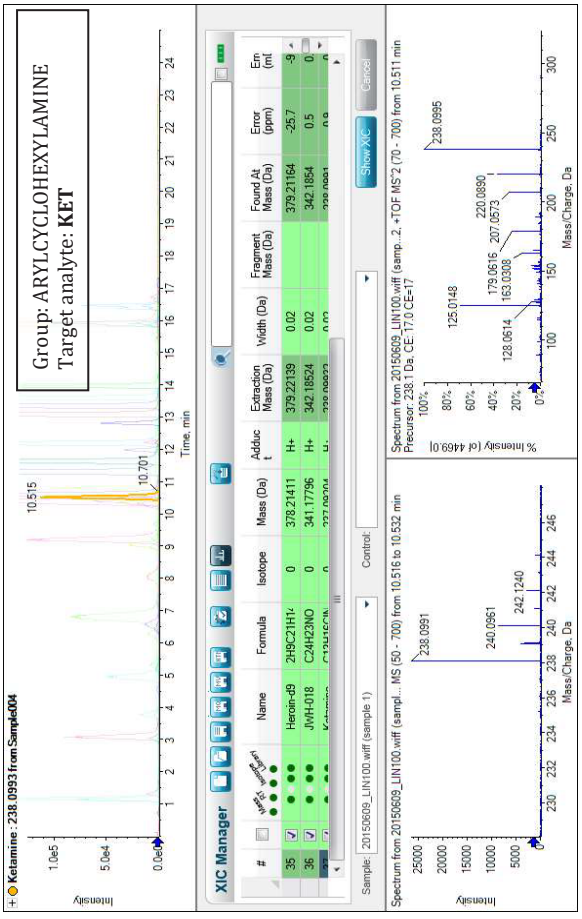
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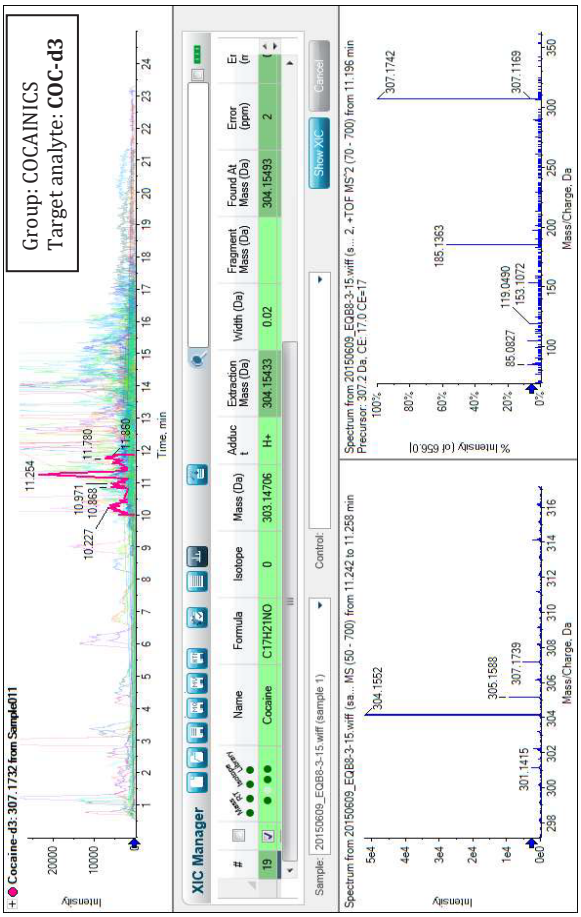
Target analyte: KET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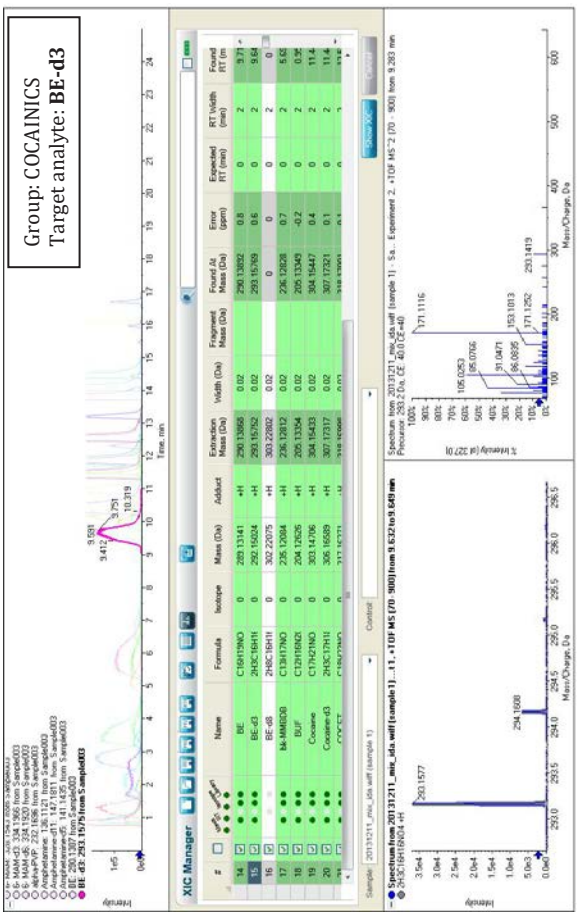
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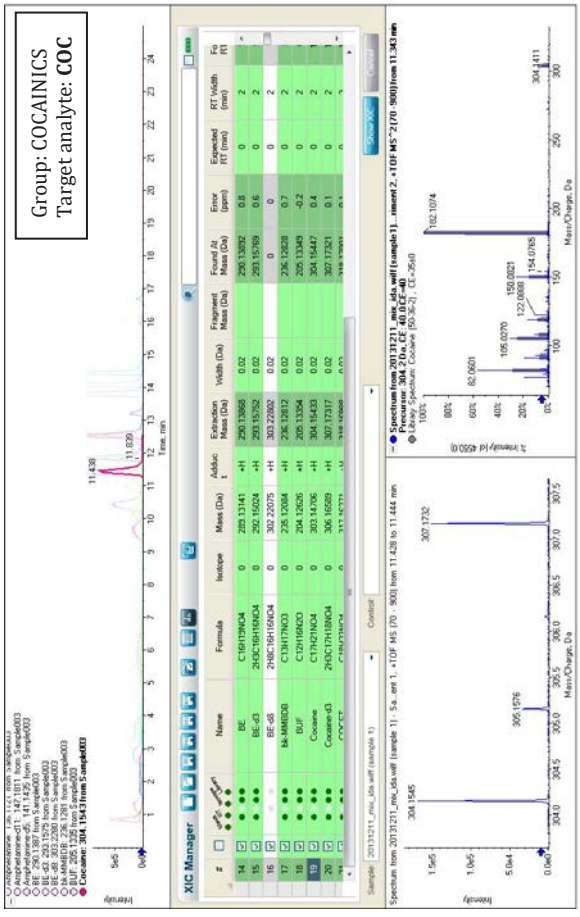
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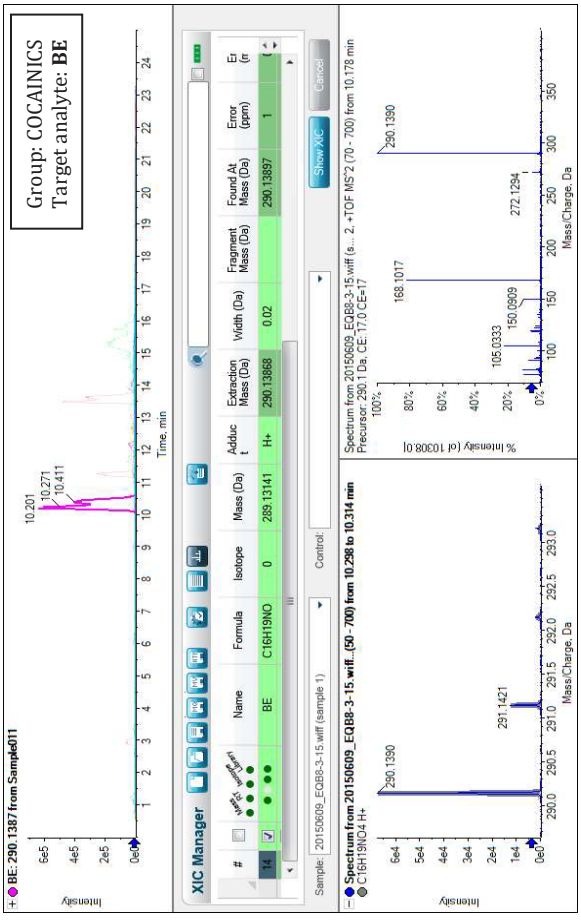
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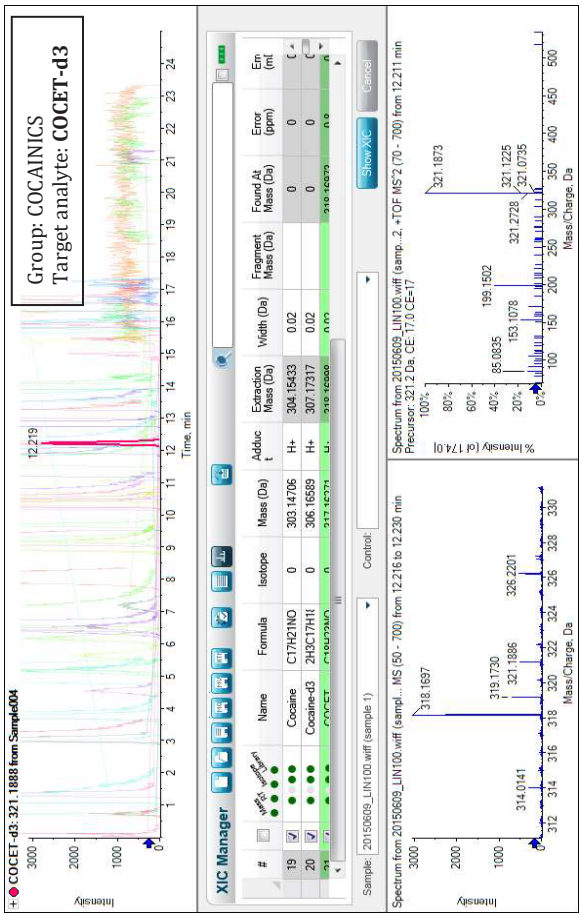
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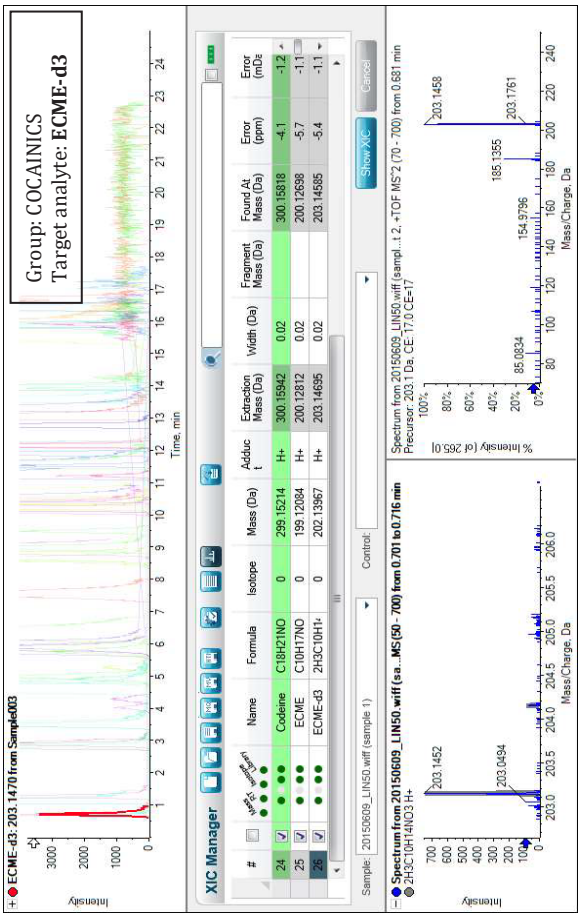
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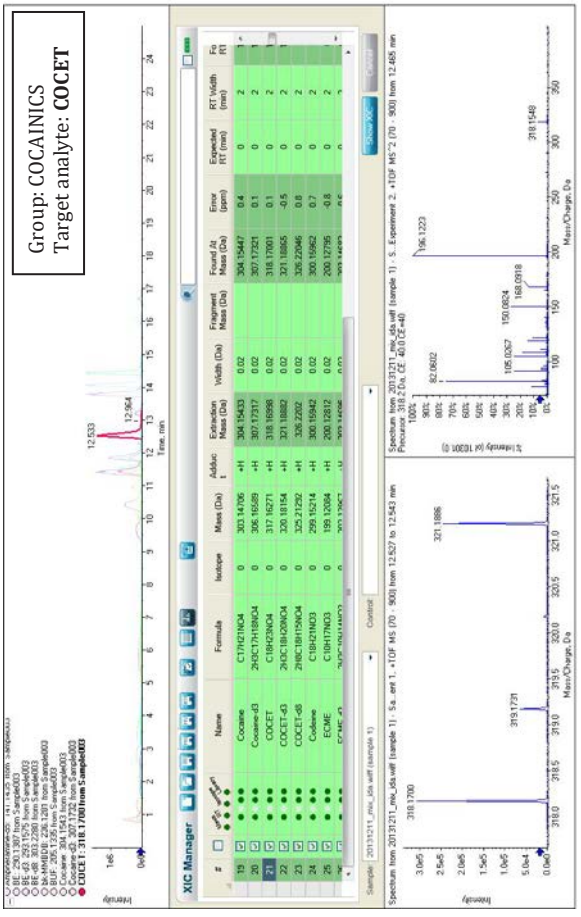
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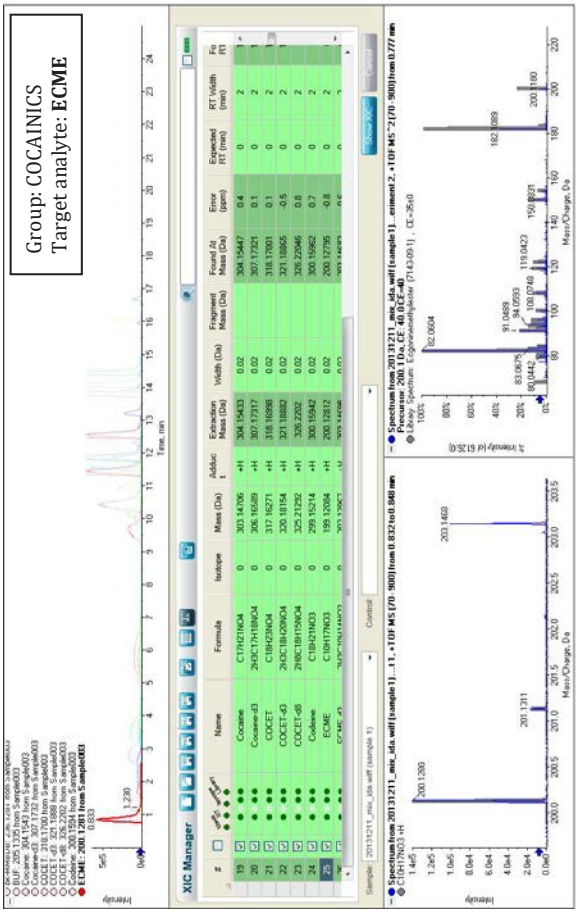
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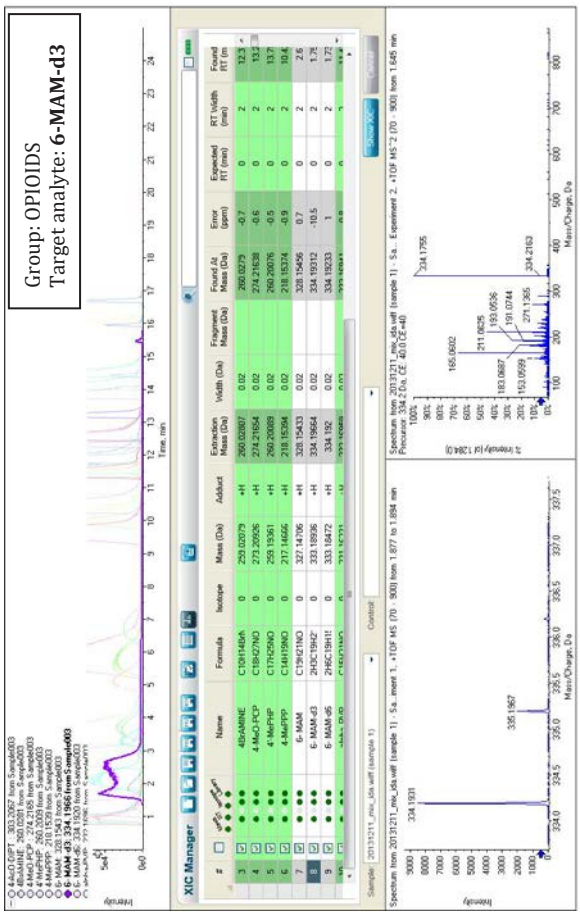
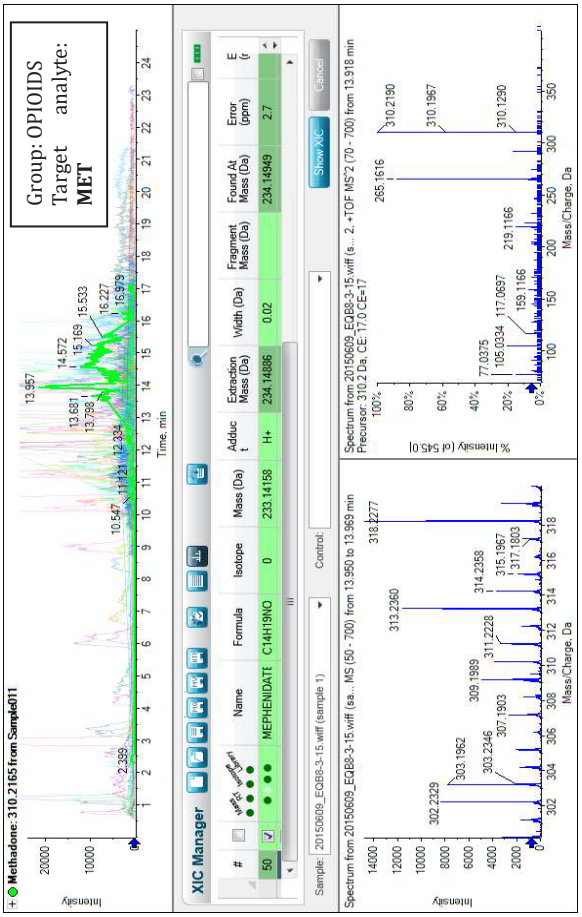
Target analyte: ECME


Group: OPIOIDS
Target analyte: 6-MAM-d3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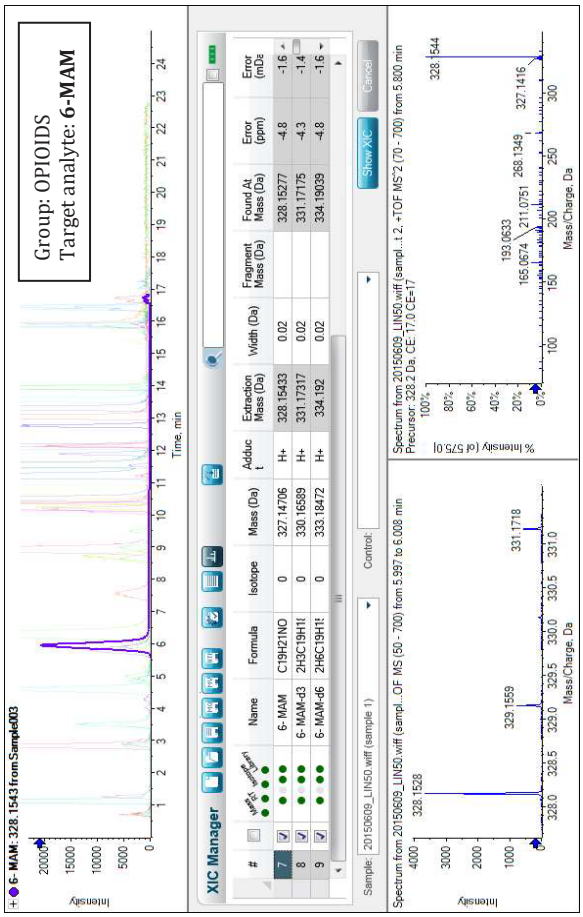
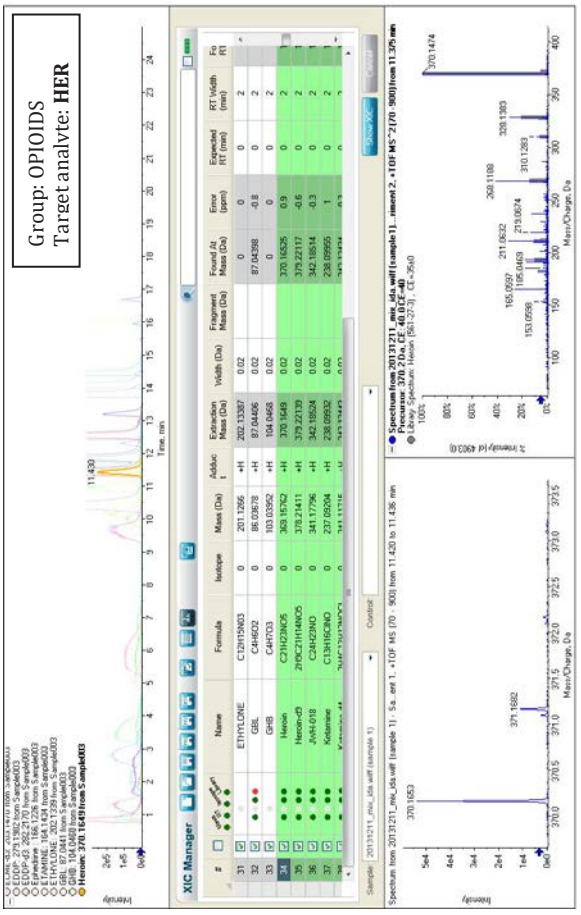
Target analyte:
MET


Group: OPIOIDS
Target analyte: 6-MAM

Group: OPIOIDS
Target analyte: COD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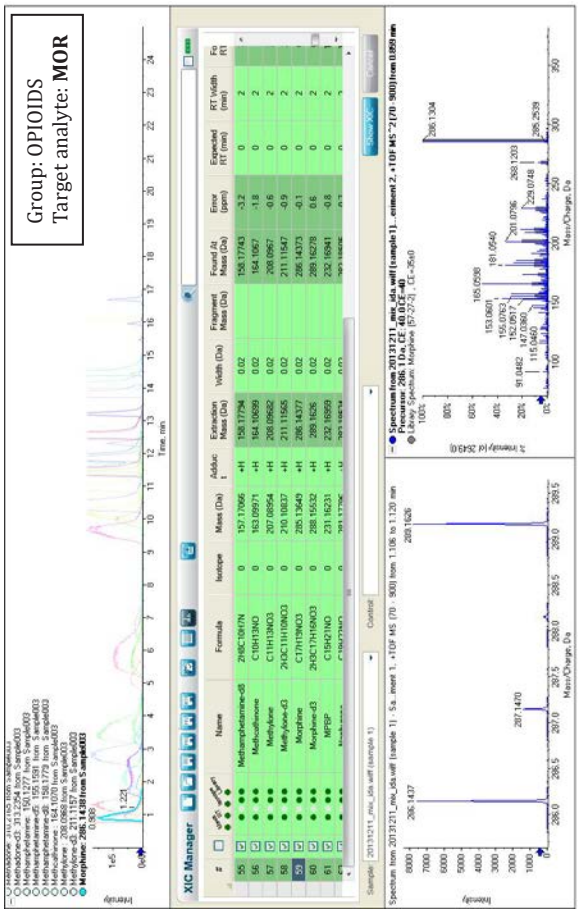
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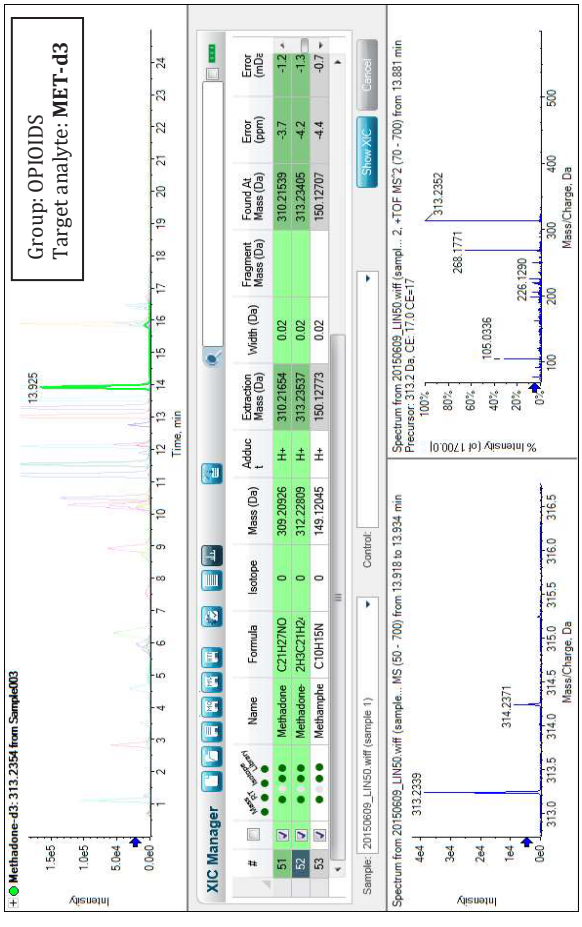
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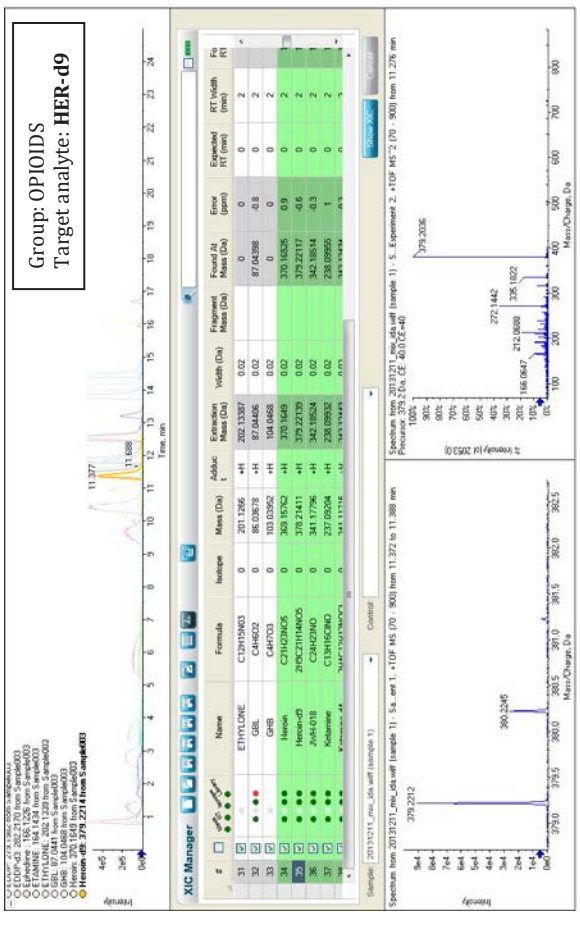
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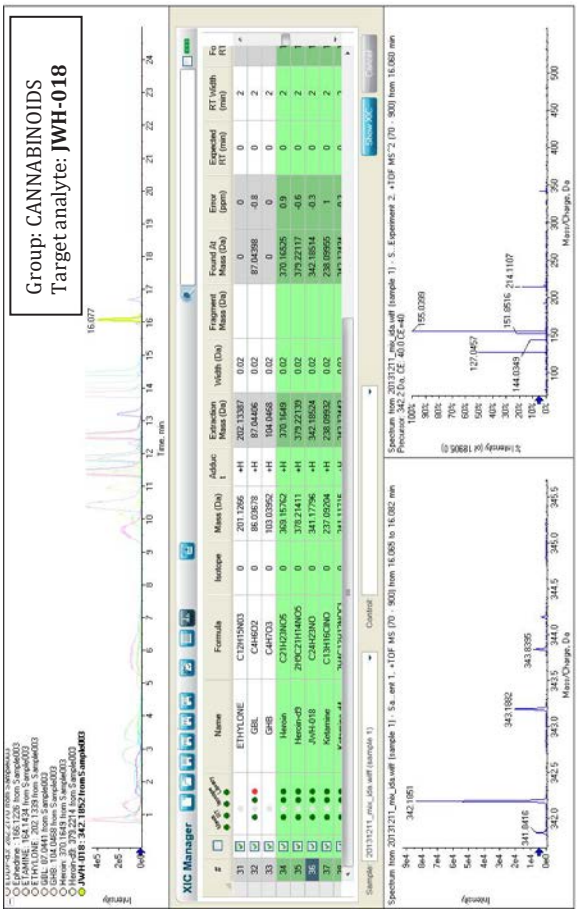
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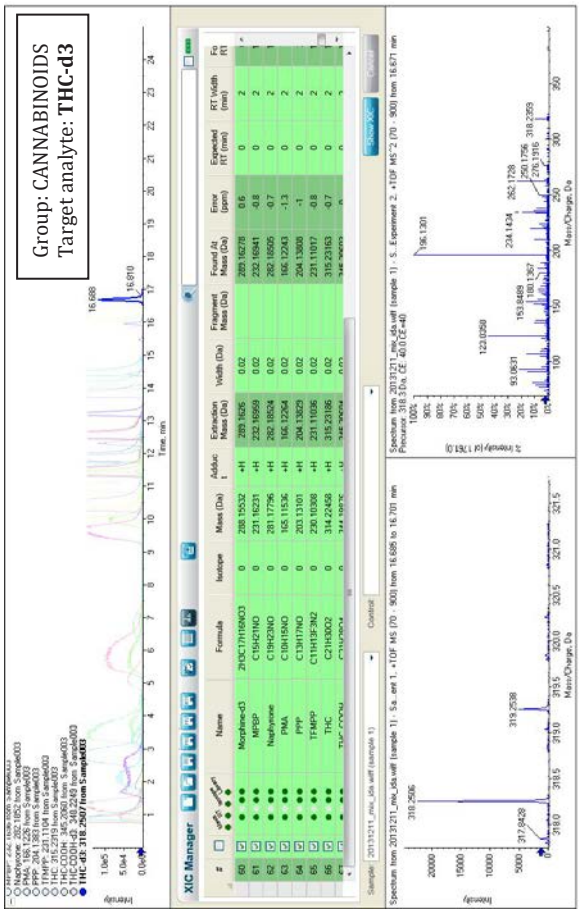
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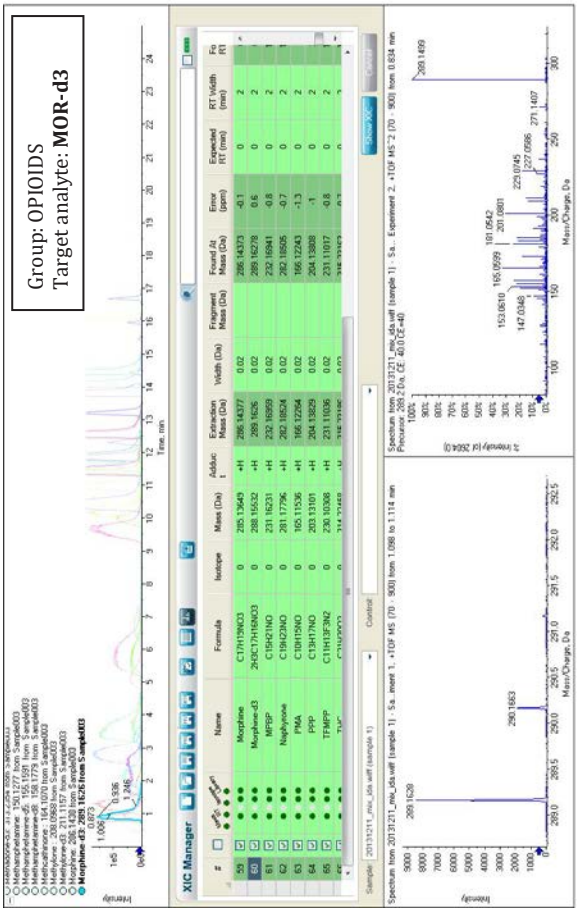
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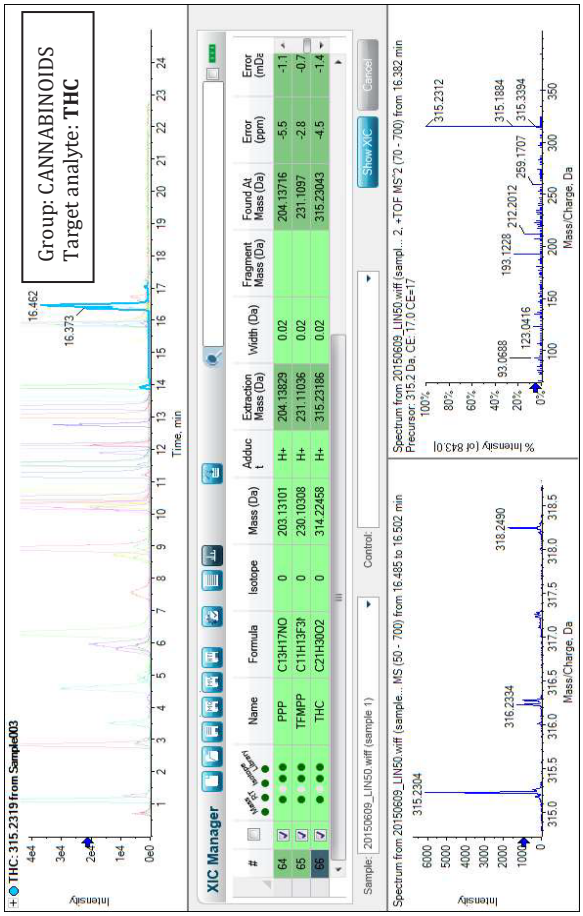
Target analyte: MOR-d3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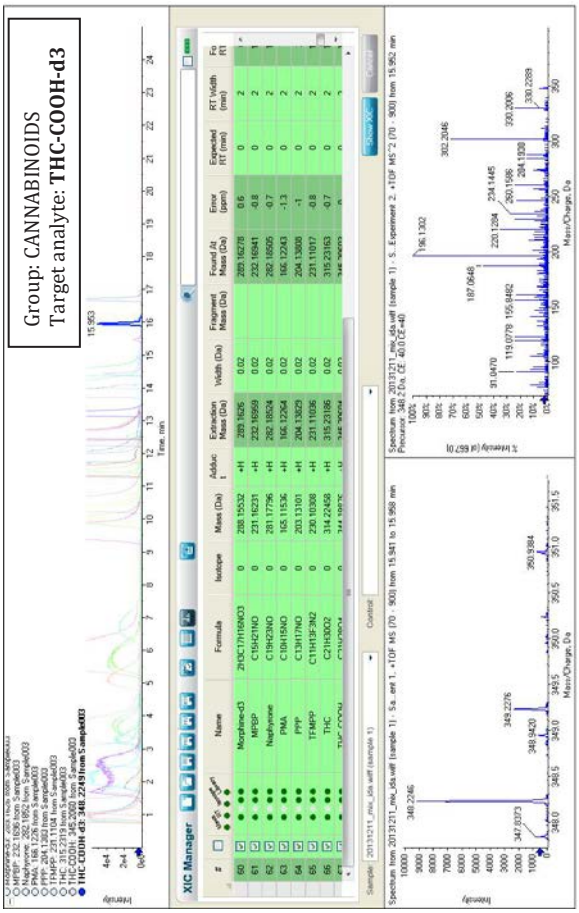
Target analyte: THC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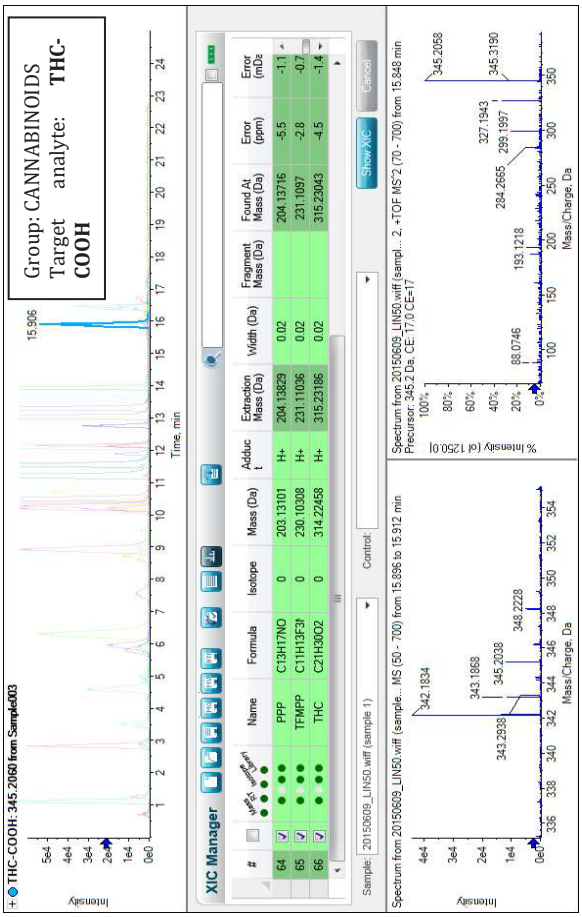
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Target analyte: THC-COOH

PUBLICACIÓN CIENTÍFICA 4

Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight

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Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of -flight

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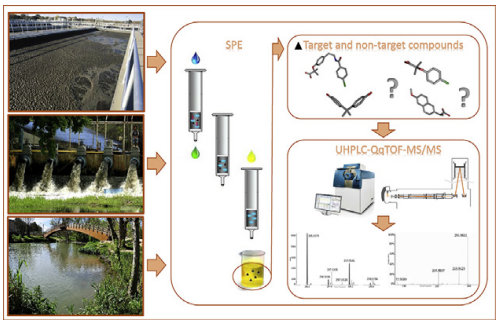
GRAPHICAL ABSTRACT



ABSTRACT

Pharmaceuticals, illicit drugs and personal care products are emerging contaminants widely distributed in water. Currently, a number of solid-phase extraction (SPE) procedures followed by liquid chromatography tandem mass spectrometry (LC–MS/MS) have been reported. However, target analysis of selected compounds is commonly used whereas other related contaminants present in the sample remain invisible. Carmona et al. [1] described a method for determining 21 emerging contaminants by LC–MS/MS with improved mobile phases. We tested this protocol in combination with high resolution mass spectrometry using a quadrupole time-of-flight (QqTOF) instrument to get a wide non-target screening approach in order to have a broader scope and more practical method for detecting licit and illicit drugs and personal care products than traditional target methods. The essential points in the method are:

- The screening capabilities of QqTOF (ABSciex Triple TOF™) are used for detecting and identifying non-target pharmaceuticals and a large number of other emerging contaminants in water.

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- The quantitative features of the instrument, the Achilles heel of the QqTOF mass spectrometers, are established for few selected compounds.
- The method may be applied to identify a large number of emerging contaminants in water. However, pre-validation will be needed to quantify them.

ARTICLE INFO
Method name: Non-target screening by SPE and UHPLC quadrupole time-of-flight
Keywords: High resolution mass spectrometry, UHPLC, emerging contaminants, water, identification, quantification
Article history: Available online 13 April 2016

Method details

Many different types of pollutants have been found in environmental compartments as water. Licit and illicit drugs or personal care products are some of the so-called emerging contaminants extensively used by humans [1,2]. A number of analytical methods are already available [3–9] to determine emerging contaminants in environmental matrices at low concentrations. However, these methods are only reported for one type of instrument. In this study, we proposed a procedure to analyse pharmaceuticals, illicit drugs, personal care products and others contaminants on different water matrices through a common method for a triple quadrupole (QqQ) and a quadrupole time-of-flight (QqTOF) mass spectrometers.

Reagents and materials

Acetaminophen, bezafibrate, bisphenol A, butylparaben, chloramphenicol, clofibric acid, diclofenac, ethylparaben, flufenamic acid, gemfibrozil, ibuprofen, indomethacin, methylparaben, naproxen, propylparaben, salicylic acid, thiamphenicol, triclocarban, triclosan and warfarin from Sigma-Aldrich (The Woodlands,Texas, USA) and tetrahydrocannabinol (THC) and 11-nor-9-carboxy-<DELTA>9-tetrahydrocannabinol (THC-COOH) from LoGiCal (Luckenwalde, Germany) were used as target analytes for QqQ analysis. Calibration standards were prepared by serial dilution of the mixed working solution. Stock and working solutions were stored at −20 °C in the dark [10].

Water used for preparation of calibration standards and LC–MS mobile phase was purified by an Elix Milli-Q system (Millipore, Billerica, MA, USA). Methanol was purchased from Panreac (Castellar del Vallès, Barcelona, Spain) and formic acid was purchased from Amresco (Solon, OH, USA). Ammonium fluoride was acquired from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany).

Extraction procedure

(1) Vacuum filter the samples (250 mL) through 0.45 μm retention capacity glass fiber filter of 90 mm diameter by Advantec (Toyo Roshi Kaisha, Ltd., Japan) using a Bücher funnel (with the filter) over a 250 mL Kitasato flask with 400 mbar h^{-1} Pa^{-1} of vacuum, to remove solid particles before the solid phase extraction (SPE).
(2) Put the Phenomenex Strata-X 33u Polymeric Reversed Phase (200 mg/6 mL) cartridges (Phenomenex, Torrance, Ca, USA) into a 12 port vacuum manifold Supelco Visiprep 57030-U of Sigma-Aldrich (St. Louis, MO, EEUU).
(3) Condition the cartridge with 6 mL methanol and 6 mL of Milli-Q water both with 400 mba h^{-1} Pa^{-1} vacuum.
(4) Pass the samples through the cartridges under previous vacuum at a flow rate of 10 mL min^{-1}.
(5) Wash the cartridges with 6 mL of Milli-Q water.
(6) Dry the cartridges under vacuum for 15 min.
(7) Elute the analytes on a 15 mL Falcon tube VWR (Radnor, PA, USA) with 6 mL of methanol and then 3 mL of a methanol–dichloromethane solution (1:1, v/v) at gravity flow.

(8) Evaporate the extracts to dryness at 40 °C using a combined sample concentrator model SBHCONC/1 and a heating plate model SBH130D/3 both manufactured by Stuart® (Stafford, UK).
(9) Redissolve the residue in 1 mL of water-methanol (70:30, v/v) by agitation and ultrasonication for 1 min and pass the extract to 2 mL amber vials with stoppers 99 mm + Septum Sil/PTFE, both manufactured by Análisis Vínicos S.L. (Tomelloso, Spain).

UHPLC-QqTOF-MS/MS conditions

The chromatography was performed with an Agilent 1260 Infinity (Agilent, Waldbronn, Germany) using an Agilent Poroshell EC-C18 maintained at temperature of 30 °C. A constant flow rate of 0.2 mL min^{-1} was used. The mobile phase consists of two solvents, 2.5 mM ammonium fluoride in methanol (as organic solvent) and 2.5 mM ammonium fluoride in water (as aqueous solvent). The UHPLC system was coupled to a hybrid QqTOF ABSciex Triple TOF™ 5600 (Framingham, MA, USA). The MS acquisition was performed using negative ionization (NI) and scan mass spectra between m/z 100–700 with the Turbo Ionspray source. The MS parameters were: ion spray voltage, 5000 V; declustering potential (DP), 120 V; collision energy (CE), 10; temperature 400 °C with curtain gas (CUR) 25 (arbitrary units); ion source gas 1 (GS1) 50 and ion source gas 2 (GS2) 50. The QqTOF-MS/MS instrument was calibrated after every three samples using external reference compounds. The MS/MS acquisition was also performed using information-dependent acquisition (IDA) following operating parameters: declustering potential two (DP2), 110 V; ion release delay (IRD), 67 V; ion release width (IRW), 25 V; IDA MS/MS was performed at a fixed CE of 40 V, ions that exceeded 100 cps and ion tolerance of 50 mDa (isotopes higher than 4 Da were excluded). Data acquisition and processing was carried out using software Analyst (Framingham, MA, USA), Peak View 1.0 with the application XIC manager and MultiQuant 2.0.

Sampling

The developed method was applied to 21 influent and 21 effluent samples collected from three wastewater treatment plants (WWTPs) of metropolitan area of Valencia and 25 surface waters from Túria River. Wastewater samples were 24-h composite samples and river samples were grab ones. All samples were stored in polyethylene terephthalate (PET) bottles and once arrived at the laboratory, immediately frozen at −20 °C until analysis to prevent degradation of contaminants.

Validation of the analytical method

Validation of the analytical method was performed partly according to the Commission Decision 2002/657/EC [11] and partly to the Eurachem guide [12] on that subject since none of them has a binding nature for water contaminants.

Table 4.1 shows limit of quantification (LOQ), matrix effect (ME), recovery and relative standard deviation (RSD) obtained by UHPLC-QqTOF determination. The method provides LOQ between 1 and 150 ng L^{-1}, recoveries from 39% to 115%, matrix effects ranged from 6 to −52% and relative standard deviations (RSD) lower than 21%. The linearity was determined by calibration curves from LOQ-5000 ng L^{-1} in water-methanol (70:30) or as a matrix matched standards, with linear coefficients of determination (R^2)≥0.99, except for salicylic acid (R^2)≥0.98. Table **S4.1** in Supplementary information depicts these parameters for UHPLC-QqQ.

Table 4.2 shows the quantification of the selected analytes in the different water samples, as mean value ± RSD using QqQ and QqTOF instruments. The quantification of the detected compounds in the three matrices with QqQ was carried out according to the instrumental conditions previously reported [1] (see Table **S4.2** in Supplementary information). The quantification of detected compounds with QqTOF was performed using MultiQuant 2.0 software. The results of QqQ and QqTOF were very similar, which confirms that the method is valid for both.

Table 4.3 presents, mass (Da), adduct, extraction mass (Da), mass error (ppm), retention time (RT) and intensity of the selected compounds (spiked Milli-Q water with 100 ng L^{-1}). The identification of

Table 4.1
Method performance parameters: limit of quantification (LOQ, ng L^{-1}), absolute recoveries (%), method repeatability (RSD, %) and matrix effect (ME, %) using QqTOF for effluent, influent and river water samples.

Analyte	WWTP Influent				WWTP Effluent				River water			
	LOQ (ng L^{-1})	Recovery (%)	RSD (%)	ME (%)	LOQ (ng L^{-1})	Recovery (%)	RSD (%)	ME (%)	LOQ (ng L^{-1})	Recovery (%)	RSD (%)	ME (%)
Acetaminophen	30	84	15	−33	15	86	14	−26	15	95	10	−12
Bezafibrate	30	75	15	−32	20	78	13	−28	10	85	11	−16
Bisphenol A	20	80	19	−12	10	80	12	−10	5	89	17	−18
Butylparaben	10	79	17	−19	5	101	18	−19	5	115	12	−10
Chloramphenicol	50	62	11	−36	20	75	17	−32	20	92	10	−23
Clofibric acid	100	61	12	−41	30	70	21	−31	20	76	20	−31
Diclofenac	150	82	10	−47	40	91	15	−45	30	98	12	−15
Ethylparaben	50	81	13	−31	25	95	11	−35	20	96	18	−28
Flufenamic acid	40	71	14	−29	30	69	15	−18	5	89	15	−16
Gemfibrozil	10	61	9	−29	10	67	12	−20	10	78	17	−9
Ibuprofen	100	80	11	−32	80	92	18	−15	50	90	12	−11
Indomethacin	50	78	15	−15	50	98	10	−11	30	79	13	−2
Methylparaben	30	80	9	−33	10	90	12	−35	5	89	20	−19
Naproxen	50	71	17	−30	20	85	18	−32	30	89	17	−21
Propylparaben	50	71	21	−31	5	81	13	−24	10	102	13	−5
Salicylic Acid	100	39	10	−52	50	62	18	−39	20	61	25	−13
THC	50	48	18	−9	20	52	17	−10	10	54	19	−6
THC-COOH	10	50	9	−19	10	63	14	−19	5	62	15	6
Thiamphenicol	120	74	11	−21	100	92	19	−20	80	89	18	−7
Triclocarban	50	85	13	−19	5	79	15	−21	5	91	14	5
Triclosan	20	82	19	−10	20	91	15	2	10	76	15	−12
Warfarin	30	73	8	−11	20	84	12	−22	1	86	13	−13

Linearity: linear coefficients (R^2) were ≥ 0.99 in all cases, except for salicylic acid ($R^2 \geq 0.98$); LOQ was established as the concentration that, after extraction, gives a UHPLC peak height value 1.0×10^4; Recoveries and relative standard deviations (RSDs) of selected compounds were calculated in samples spiked at 100 ng L^{-1} subtracting the peak areas corresponding to native analytes in the sample and tested in quintuplicate; Matrix effect was evaluated by comparing the slope of the calibration curves obtained for spiked influent, effluent or surface water extracts with the slope of that obtained for standard prepared in water-methanol (70:30, v/v) spiked at the same level.

target and non-target was carried out against the XIC manager Table with data of 1212 pharmaceuticals, 546 pesticides, 378 polyphenols and 233 mycotoxins. Furthermore, a total of 86 ± 9 pharmaceuticals, 2 ± 1 pesticides and 14 ± 3 other compounds were detected in influent samples; 45 ± 14 pharmaceuticals, 1 ± 1 pesticides and 7 ± 3 other compounds were detected in effluent samples, and 20 ± 6 pharmaceuticals, 1 ± 1 pesticides and 5 ± 3 other compounds in river water samples. Fig. 4.1 illustrates the identification of acetaminophen (paracetamol) and Fig. 4.2 of the non-selected hydrochlorothiazide to show the identification system capabilities. Fig. **S4.1** in Supplementary information shows the extracted ion chromatogram of all substances present in water and the non-target compound identification of theophylline in influent wastewater sample.

Additional information

Background

There are hundreds, even thousands of emerging contaminants that can occur in water. Traditionally, the scheme used for their determination involves generic sample preparation procedures able to extract almost any of them, and target determination for the unique and highly specific detection of the selected contaminant(s) [3–5]. This scheme is time-consuming (ca. 30 min each chromatographic run for a specific group of contaminants) and do not have versatility to detect unexpected emerging contaminants not selected for the target analysis. Currently, there are some reports of non-target detection through high resolution mass spectrometry that provide full scan

Table 4.2
Comparison of the quantitative results obtained using the ABSciex TripleTOF™ 5600 (QqTOF) and a more traditional triple quadrupole (QqQ) for influent, effluent and river water samples.

Compounds[a]	Sample 1 Influent (ng L^{-1})[b]		Sample 2 Effluent (ng L^{-1})[b]		Sample 3 River Water (ng L^{-1})[b]	
	QqTOF	QQQ	QqTOF	QQQ	QqTOF	QQQ
Acetaminophen	2114	2497	31	21	139	177
Bezafibrate	35	47	11	15	12	7
Bisphenol A	495	571	96	72	36	41
Butylparaben	35	22	n.d.	n.d.	7	5
Chloroamphenicol	n.d.	n.d.	n.d.	n.d.	62	68
Clofibric acid	12	7	n.d.	n.d.	n.d.	n.d.
Diclofenac	296	331	109	173	39	33
Ethylparaben	99	113	49	71	n.d.	6
Flufenamic acid	75	90	39	48	29	22
Gemfibrozil	105	155	n.d.	5	31	34
Ibuprofen	1796	1978	n.d.	n.d.	159	153
Indomethacin	n.d.	7	n.d.	18	n.d.	n.d.
Methylparaben	259	331	121	99	19	24
Naproxen	2963	3327	21	10	38	36
Propylparaben	494	519	36	45	11	12
Salicylic acid	596	778	n.d.	n.d.	29	22
THC	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
THC-COOH	409	592	n.d.	n.d.	21	23
Thiamphenicol	n.d.	n.d.	n.d.	n.d.	n.d.	10
Triclocarban	n.d.	7	n.d.	n.d.	n.d.	n.d.
Triclosan	752	926	n.d	n.d.	n.d.	n.d.
Warfarin	n.d.	11	29	31	33	54

n.d.: non-detected.
[a] Only analytes that occur in any of the samples.
[b] Average (SD) n = 3.

information as well as compound fragmentation (any *m*/*z* signal from the sample extract) [2,8]. However, high resolution mass spectrometer can provide inaccurate quantification [8] or enough

Table 4.3
Experimental parameters used for the identification of the target analytes (n = 5).

Name	Mass (Da)	Adduct	Extraction Mass (Da)	Found at mass (Da)	Error ppm	Error (mDa)	Found at RT (min)	Intensity
Acetaminophen	151.06333	−H	150.05605	150.05612	0.4	0.3	1.12	35326
Bezafibrate	361.10809	−H	360.10427	360.10409	−0.8	−0.2	14.36	40634
Bisphenol A	228.11504	−H	227.11496	227.11431	−2	−0.7	14.86	73687
Butylparaben	194.09430	−H	193.09421	193.09438	0.8	0.2	13.31	70035
Chloramphenicol	322.01233	−H	321.01129	321.01174	1.2	0.4	10.38	63257
Clofibric acid	214.03967	−H	213.03037	213.02899	−4.2	−1.4	9.89	55963
Diclofenac	295.01669	−H	294.01596	294.01617	0.6	0.2	15.87	75981
Ethylparaben	166.06299	−H	165.06196	165.06323	3.3	1.3	12.36	62257
Flufenamic acid	281.06636	−H	280.05909	280.05942	1.2	0.3	14.63	45704
Gemfibrozil	250.15689	−H	249.14962	249.1498	0.7	0.2	14.59	64434
Ibuprofen	206.13068	−H	205.1234	205.12357	0.8	0.2	14.52	70035
Indomethacin	357.07678	−H	356.07536	356.07640	2.9	2.9	16.25	59363
Methylparaben	152.04735	−H	151.04631	151.04657	0.9	0.9	9.64	61259
Naproxen	230.09430	−H	229.09411	229.09489	2.6	2.6	13.91	79632
Propylparaben	180.07864	−H	179.07796	179.07803	0.4	0.4	14.47	42963
Salicylic acid	138.03169	−H	137.03165	137.03172	0.4	0.4	2.56	49332
THC	314.22458	−H	313.2173	313.21728	−0.1	0	16.11	44379
THC-COOH	344.19876	−H	343.19148	343.19193	1.3	0.4	14.63	73637
Thiamphenicol	355.00479	−H	354.00432	354.00499	1.5	1.5	2.67	75336
Triclocarban	313.97806	−H	312.97124	312.97111	−0.4	−0.4	15.63	48525
Triclosan	287.95117	−H	286.90985	286.91012	1	1	16.57	71225
Warfarin	308.10486	−H	307.10362	307.10348	−0.4	−0.4	10.78	79325

RT: retention time.

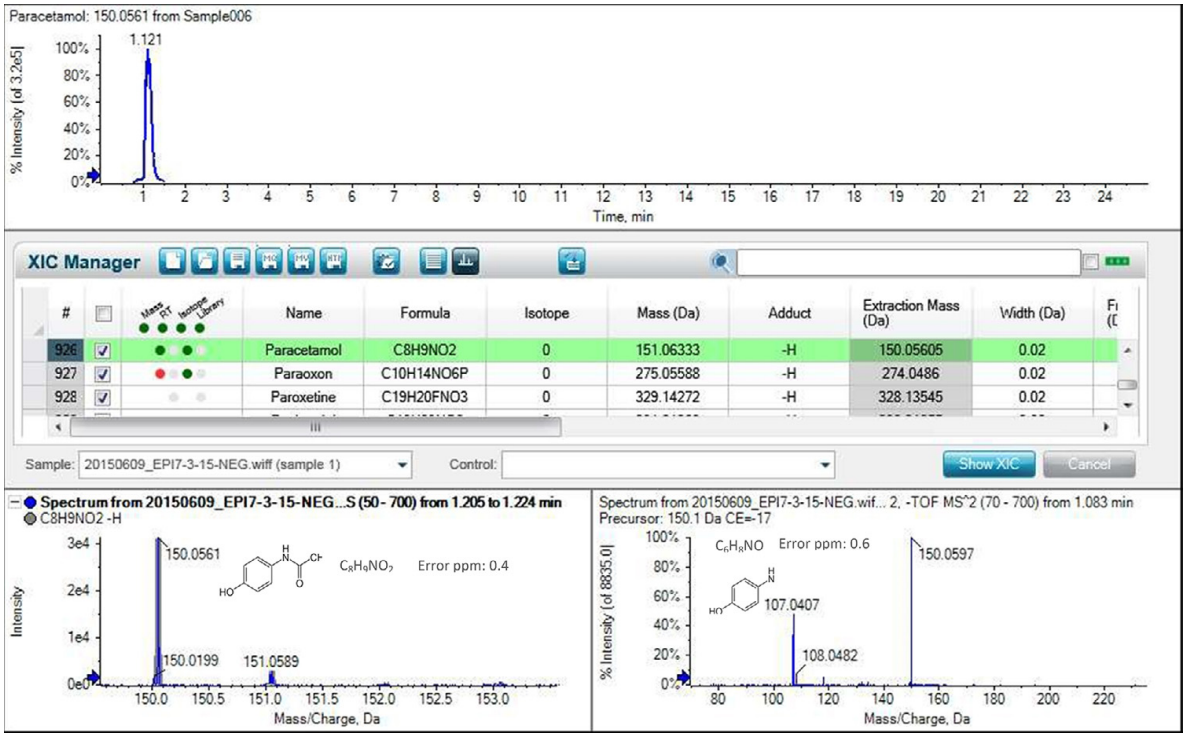

Fig. 4.1. MS and MS/MS Spectra of target analyte acetaminophen (paracetamol).

sensitivity [2]. Latest generation instruments have improved their quantification possibilities as well as the identification capabilities of any unexpected substance by the application information dependent acquisition (IDA) modes that automatically provide MS/MS spectra of the most intense precursor ions (without previous selection) as an additional confirmation of the detected compounds [2].

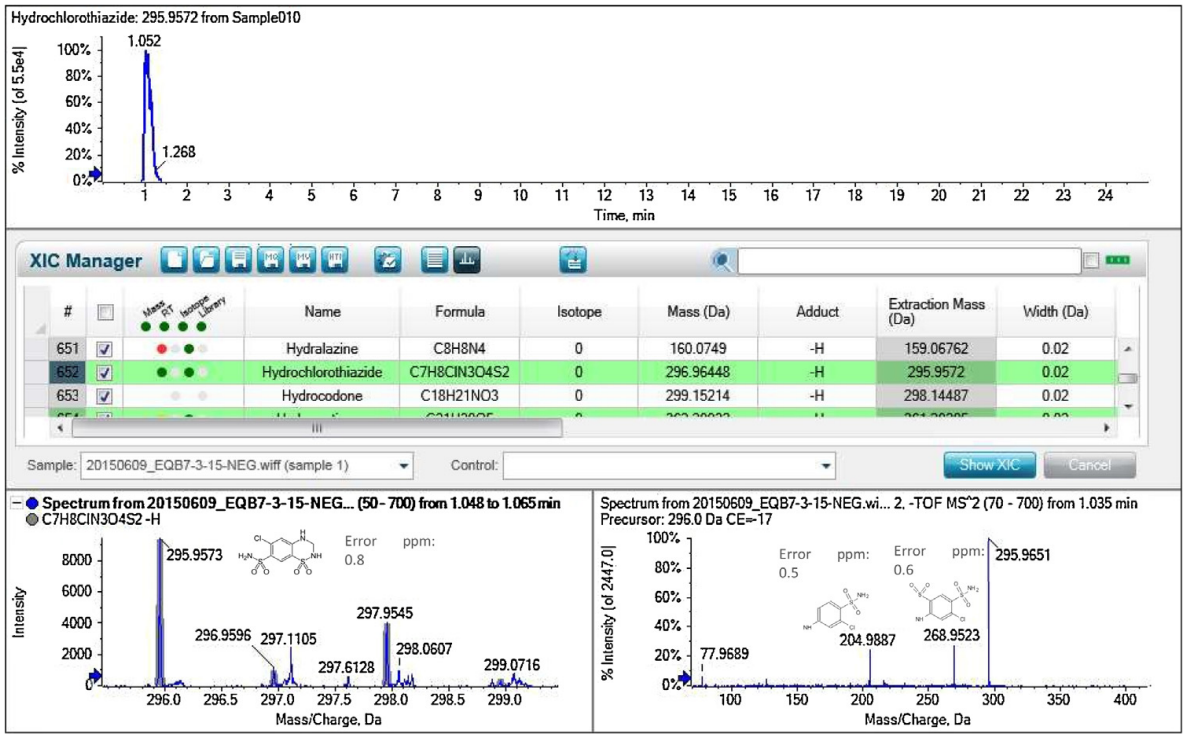

Fig. 4.2. MS and MS/MS Spectra of non-target analyte hydrochlorothiazide.

The few examples of these broad screening systems are mostly focus on the positive ionization mode because there are more contaminants that ionized in positive mode and their MS sensitivity is higher. When mass spectrometry is combined with liquid chromatography (recommended for polar compounds as the emerging contaminants) the commonly used additives of the mobile phases (volatile salts and acids) enhanced the ionization in the positive ionization mode and inhibited it in the negative ionization one. Acidic contaminants, commonly better ionized by negative ionization are more difficult to detect and frequently the sensitivity does not reach the low levels emerging contaminants are present in water. Recently, Petrie et al. [9] demonstrated a substantial improvement of ionization efficiency in negative ionization mode by using NH_4F enriched mobile phase to metabolomics studies. Our previously reported method using NH_4F as mobile phase additive instead of more conventional substances also improved the ionization efficiency of the 21 selected compounds in a reproducible way using a triple quad instrument [1]. These results were recently confirmed for wide range of compounds [10]. Our current study proves that the addition of NH_4F to the mobile phase instead of more conventional ammonium formate is also successful for the simultaneous determination of acidic contaminants in water by UHPLC-QqTOF [13,14] increasing sensitivity and quantification capabilities. The strong basicity of the fluoride anion (F^-) in the gas phase increases deprotonation of basic analytes.

The results showed good agreement between both systems for the analysed samples. For QqQ, naproxen was the pharmaceutical at highest concentration (3327 ng L^{-1}) at the influent of the WWTPs which was in a lower concentration at the effluent (10 ng L^{-1}). Indomethacin, clofibric acid and triclocarban were the lowest detected with 7 ng L^{-1} in influent samples. Regarding effluent samples, the highest detected concentration was diclofenac with 173 ng L^{-1}, being the gemfibrozil the compound with the lowest (5 ng L^{-1}). Finally, for river waters, the concentration of target analytes was, in general, lower than WWTPs samples being the compound in major concentration the acetaminophen with 177 ng L^{-1} and ibuprofen with 153 ng L^{-1}. Concerning the concentration calculated with QqTOF, the mean concentration levels detected in influent samples ranged from 12 ng L^{-1} (clofibric acid) to 2963 ng L^{-1} (naproxen) being naproxen the most detected compound as in the case of QqQ. In the effluent the highest concentrations were methylparaben (121 ng L^{-1}) followed by diclofenac (109 ng L^{-1}). In river waters the concentration levels ranged from 7 ng L^{-1} (butylparaben) to 159 ng L^{-1} (ibuprofen). These results show a good correlation between both techniques as in our previous paper [3].

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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.mex.2016.04.004.

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Supplementary information

Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography Quadrupole Time-of-Flight

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Table S4.1. UHPLC-QqQ-MS/MS conditions for target compounds.

Compound	Molecular formula	RT (min)	SRM1	Frag/CE(V)	SRM2	Frag/CE(V)
Acetaminophen	$C_8H_9NO_2$	12.1	150 > 108	88/14	--	--
Bezafibrate	$C_{19}H_{20}ClNO_4$	13.3	360 > 274	106/10	360 > 154	106/22
Bisphenol A	$C_{15}H_{16}O_2$	13.8	227 > 212	138/14	227 > 133	138/25
Butylparaben	$C_{11}H_{14}O_3$	14.6	193 > 137	122/10	193 > 92	122/10
Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	8.4	321 > 152	128/10	321 > 176	128/10
Clofibric acid	$C_{10}H_{11}ClO_3$	9.2	213 > 127	76/1	213 > 35	76/33
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	14.5	294 > 250	88/10	294 > 178	88/22
Ethylparaben	$C_9H_{10}O_3$	11.8	165 > 92	103/10	165 > 137	103/22
Flufenamic Acid	$C_{14}H_{10}F_3NO_2$	15.1	280 > 236	106/10	280 > 176	106/30
Gemfibrozil	$C_{15}H_{22}O_3$	16.5	249 > 121	88/10	249 > 127	88/20
Ibuprofen	$C_{13}H_{18}O_2$	14.8	205 > 161	68/2	205 > 159	68/12
Indomethacin	$C_{19}H_{16}ClNO_4$	15.0	356 > 296	98/10	356 > 282	98/22
Methylparaben	$C_8H_8O_3$	9.1	151 > 92	93/10	151 > 136	93/18
Naproxen	$C_{14}H_{14}O_3$	12.0	229 > 170	88/10	229. > 169	88/26
Propylparaben	$C_{10}H_{12}O_3$	13.4	179 > 92	112/10	179 > 137	112/22
Salicylic Acid	$C_7H_6O_3$	2.1	137 > 93	86/10	137 > 64	86 / 30
THC	$C_{21}H_{30}O_2$	17.5	313 >191	186/26	313 >245	186/26
THC COOH	$C_{21}H_{28}O_4$	13.6	343>245	166/18	343 >299	166/20
Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	2.3	354 > 290	128/10	354 > 64	128/74
Triclocarban	$C_{13}H_9Cl_3N_2O$	16.8	313 > 160	86/10	313 > 126	86/10
Triclosan	$C_{12}H_7Cl_3O_2$	16.9	287 > 35	98/14	289 > 35	98/13
Warfarin	$C_{19}H_{16}O_4$	11.8	307 > 161	136/10	307 > 117	136/30

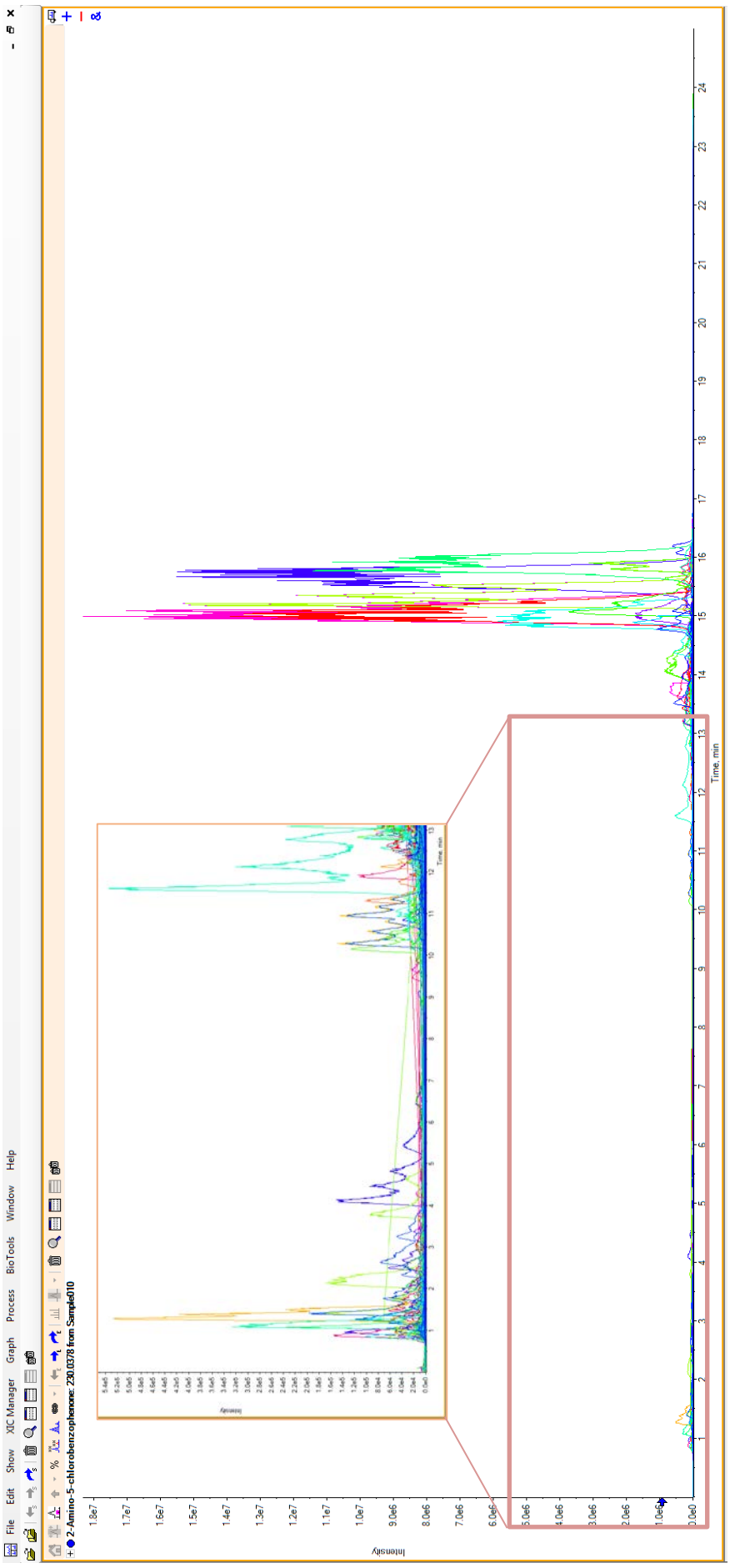
RT: Retention Time; SRM: Selected Reaction Monitoring; Frag: Fragmentor; CE: Collision Energy

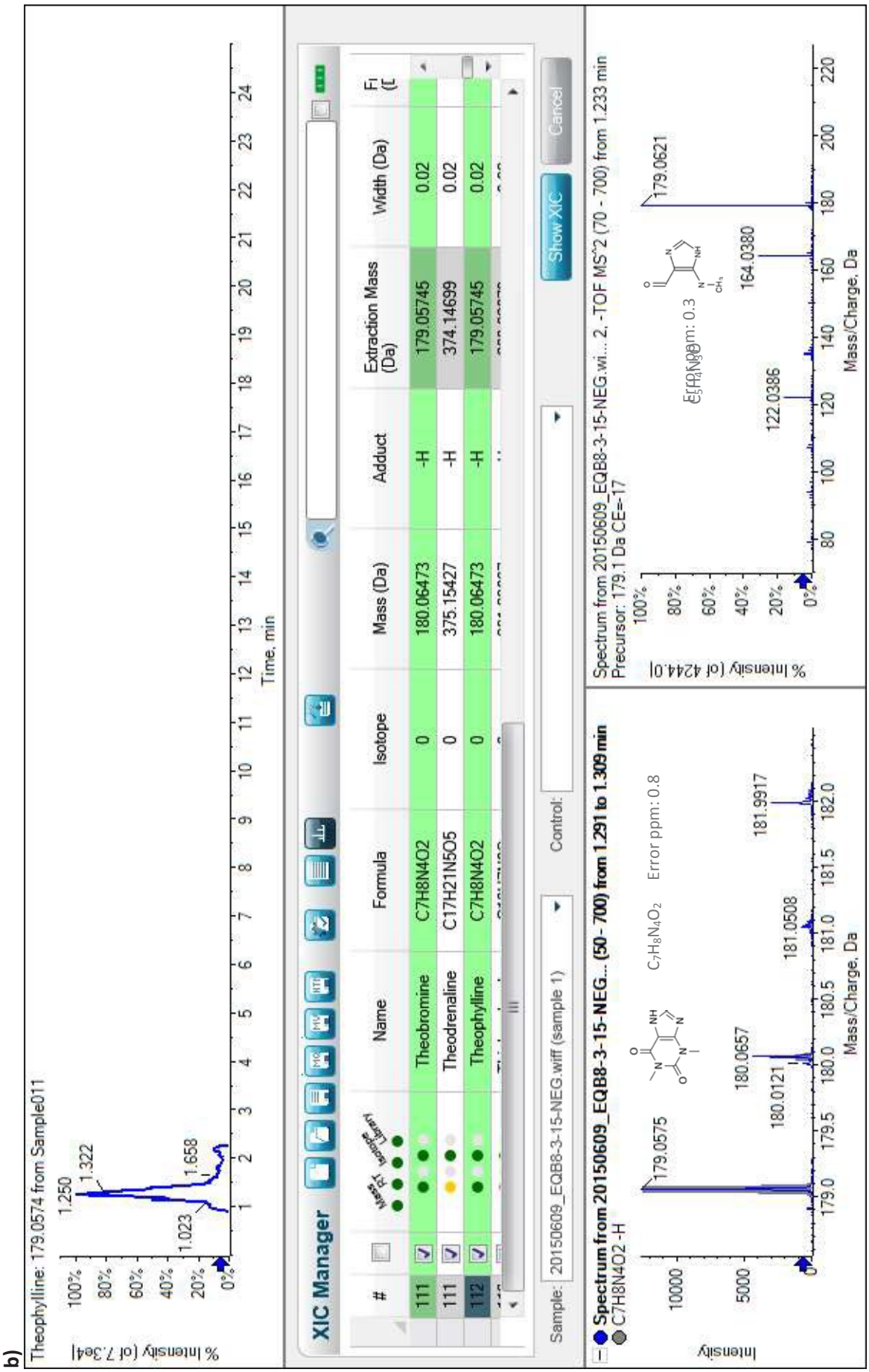
Table S4.2. Method performance parameters: limit of quantification (LOQ, ng L^{-1}), absolute recoveries (%), method repeatability (RSD, %) and matrix effect (ME %) of QqQ for effluent, influent and River water samples.

Analyte	WWTP Influent				WWTP Effluent				River water			
	LOQ (ng L^{-1})	Recovery (%)	RSD (%)	ME (%)	LOQ (ng L^{-1})	Recovery (%)	RSD (%)	ME (%)	LOQ (ng L^{-1})	Recovery (%)	RSD (%)	ME (%)
Bezafibrate	5	71	18	-25	1	76	16	-18	1	80	11	-10
Bisphenol A	2	80	15	-18	1	83	10	-20	1	82	17	-10
Butylparaben	0.7	86	15	-25	0.5	98	15	-19	0.8	105	12	-7
Chloramphenicol	15	62	12	-40	6.5	69	15	-26	5	80	10	-25
Chlorfibric acid	10	71	13	-45	3	79	20	-30	2	77	20	-20
Diclofenac	18	80	16	-45	4	88	11	-40	4	100	12	-30
Ethylparaben	5	89	21	-35	1.5	99	18	-20	1	98	18	-15
Flufenamic Acid	4	70	15	-20	0.6	70	13	-7	0.5	83	15	-5
Gemfibrozil	10	65	14	-25	1.5	72	11	-7	1	77	17	0
Ibuprofen	26	87	22	-20	20	96	17	-10	15	93	12	-4
Indomethacin	15	68	14	-17	5	72	20	-8	5	75	13	-5
Methylparaben	6	86	12	-50	1	91	18	-35	1	91	20	-15
Naproxen	6	77	20	-35	2	86	13	-20	3	86	17	-12
Propylparaben	5	91	25	-30	0.5	94	12	-25	0.5	111	13	-10
Salicylic acid	15	38	13	-65	1	49	19	-40	0.5	49	25	-20
THC	2	47	20	-17	0.5	50	17	-15	0.4	55	19	2
THC COOH	1	55	15	-20	1	60	15	-15	0.6	59	15	0
Thiamphenicol	12	79	19	-40	14	82	20	-25	15	87	18	-15
Triclocarban	5	83	17	-15	0.5	89	14	-10	0.3	99	14	0
Triclosan	2	91	18	-13	5	95	15	-12	4	100	15	0
Warfarin	5	79	10	-15	1	83	13	-20	0.7	81	13	-5

Figure S4.1. a) Extracted ion chromatogram of the non-target compounds. **b)** theophylline in influent wastewater sample against the XIC manager Table with data on 1212 pharmaceuticals, 546 pesticides, 378 polyphenols and 233 mycotoxins.

a)




b)
Theophylline: 179.0574 from Sample011
% Intensity (of 7.3e4)
Time, min
XIC Manager
Name
Formula
Isotope
Mass (Da)
Adduct
Extraction Mass (Da)
Width (Da)
Theobromine
C7H8N4O2
180.06473
-H
179.05745
0.02
Theodrenaline
C17H21N5O5
375.15427
374.14699
Theophylline
C7H8N4O2
180.06473
179.05745
Sample: 20150609_EQB8-3-15-NEG.wiff (sample 1)
Control:
Show XIC
Cancel
Spectrum from 20150609_EQB8-3-15-NEG... (50 - 700) from 1.291 to 1.309 min
C7H8N4O2 -H
Error ppm: 0.8
Intensity
Mass/Charge, Da
Spectrum from 20150609_EQB8-3-15-NEG.wi... 2, -TOF MS^2 (70 - 700) from 1.233 min
Precursor: 179.1 Da CE=-17
Error ppm: 0.3
% Intensity (of 4244.0)
Mass/Charge, Da

Capítulo 3

CAPÍTULO 3

EPIDEMIOLOGÍA DE ALCANTARILLA

En el Capítulo 3 se aplican las metodologías desarrolladas en el capítulo anterior al análisis de las aguas procedentes de los influentes y efluentes de las WWTPs, para determinar la incidencia de las drogas de abuso, estimar su consumo en el área metropolitana de Valencia y establecer el rendimiento de eliminación de dichos compuestos en las principales WWTPs involucradas. Además se evalúa la toxicidad de determinadas drogas a diferentes niveles tróficos mediante estudios teóricos (ECOSAR). A partir de los datos obtenidos de los influentes se establece el consumo de drogas por parte de la población abastecida por dichas WWTPs, aplicando la *epidemiología de alcantarilla*. Asimismo se propone un método alternativo basado en el análisis de biomarcadores presentes en las aguas residuales para estimar dicha población. Este capítulo se ha estructurado en tres publicaciones científicas, las dos primeras focalizadas en la estimación de drogas y alcohol mientras que la tercera se centra en valorar los métodos para estimar la población servida por la WWTP, y donde se analizan, además de por los métodos clásicos, distintos biomarcadores recientemente propuestos como alternativas adecuadas para dicha estimación.

- Publicación científica 5. *Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)*
- Publicación científica 6. *Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker*
- Publicación científica 7. *Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study*

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Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)

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Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)

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Risk assessment

ABSTRACT

The occurrence of 8 drugs of abuse and metabolites in the influent and effluent of the 3 Wastewater Treatment Plants (WWTP) that treat wastewater from Valencia was studied in 2011, 2012 and 2013. Target drugs except 6-monoacetylmorphine (6-ACMOR) were detected in 100% of the influents. The WWTPs eliminate cocaine (COC), amphetamine (AMP), methamphetamine (MAMP) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH). Benzoylecgonine (BECG) was also efficiently eliminated (93–98%), whereas 3,4-methylenedioxymethamphetamine (MDMA) presented removal rates of 32–57% and ketamine (KET) was not eliminated. The most consumed illicit drugs, according to the estimated concentrations of each compound in the studied WWTPs, were cannabis and COC followed by KET, AMP, MAMP, MDMA and heroin. Environmental risk assessment was evaluated by calculating Risk Quotient (RQ). MDMA and KET could pose a medium risk and low risk, respectively, to the aquatic organisms. Although short-term environmental risk is not worrisome, long-term effects cannot be known exactly.

1. Introduction

Illicit drugs monitoring in waters can be useful because environmentalists manage and track environmental hazard of these substances and epidemiologists evaluate their nature, magnitude and consumption patterns. Sewage epidemiology provides an important tool for estimating local consumption by investigating mass flows of unchanged parent drugs or their metabolites. This methodology was proposed by Daughton (2001) and implemented for first time by Zuccato et al. (2005). Nowadays, it is considered a suitable alternative to estimate objectively consumption in real time (EMCDDA, 2008) and numerous research groups estimate drug of abuse use at the community level in Europe (Van Nuijs et al., 2011a), Australia (Lai et al., 2013), Canada (Metcalfe et al., 2010) and North America (Bartelt-Hunt et al., 2009; Jones-Lepp et al., 2004). Relevant examples of this approach in individual European cities have been reported in Belgium (Van Nuijs et al., 2011c, 2009a, b), Croatia (Terzic et al., 2010), France (Karolak et al., 2010; Nefau et al., 2013), Ireland (Bones et al., 2007), Italy (Zuccato et al., 2008, 2005), The Netherlands (van der Aa et al., 2013), Spain (Bijlsma et al., 2014; Boleda et al., 2009; Huerta-Fontela et al., 2008; Postigo et al., 2010), Sweden (Östman et al., 2014), Switzerland (Zuccato et al., 2008) and United Kingdom (Kasprzyk-Hordern et al., 2009a). Currently numerous research groups, including our own, are carrying out an effort to homogenize these techniques to collective level, such as the study conducted in 19 European cities comprising Valencia (Thomas et al., 2012).

In this study, 8 compounds selected for their high traditional consumption in Spain, were analyzed for 3 consecutive years (periods from one week to one fortnight depending on the year) in influents and effluents from 3 Wastewater Treatment Plants (WWTPs) that treat most of the wastewater of Valencia and its surrounding towns, located in eastern Spain. The overall consumption levels for the drugs in the different collection periods were calculated as the mean of each WWTP, while the results from the daily samples were used to evaluate weekly variations. In addition, the removal efficiencies of the WWTPs and the elimination of target analytes were evaluated and the effects of the different wastewater treatments were ascertained. The impact of treated wastewater effluent on the quality of receiving waters was

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also investigated performing an ecotoxicological risk assessment. To achieve these objectives advanced mass spectrometry techniques such as ultra high pressure liquid chromatography triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS) and ultra high pressure liquid chromatography quadrupole time-of-flight (UHPLC-QqTOF-MS/MS) were used.

2. Experimental

2.1. Reagents and materials

High purity (>99%) standard solutions of 6-acetylmorphine (6-ACMOR), amphetamine (AMP), benzoylecgonine (BECG), cocaine (COC), ketamine (KET), methamphetamine (MAMP), 3,4-Methylenedioxymethamphetamine (MDMA), at 1000 mg L^{-1} and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) at 100 mg L^{-1} in methanol were obtained from Cerilliant (Austin, TX, USA) and LGC GMBH (Luckenwalde, Germany). Working standard solutions of the analytes were prepared at different concentrations by appropriate dilution of the individual stock solutions in methanol-water (1:9, v/v). The deuterated drug analogs 6-ACMOR-d_3, AMP-d_5, BECG-d_3, COC-d_3, KET-d_4, MAMP-d_5, MDMA-d_5, and THC-COOH-d_3 – used as internal standards (IS) – were also obtained from Cerilliant and LGC GMBH at a concentration of 100 mg L^{-1} in methanol. A mixed ISs working solution at 1 mg L^{-1} was prepared in methanol. Calibration standards were prepared by serial dilution of the mixed working solution ranging from 0.25 ng mL^{-1} to 250 ng mL^{-1} and addition of ISs working solution at constant concentration of 6.25 ng mL^{-1}. These levels considering the concentration factor would be equivalent to 1–1000 ng L^{-1} and 25 ng L^{-1}, respectively, in the water samples. Stock and working solutions were stored at −20 °C in the dark.

Water used for preparation of calibration standards and UHPLC-MS/MS mobile phase was purified by an Elix Milli-Q system (Millipore, Billerica, MA, USA). Methanol was purchased from Panreac (Castellar del Vallès, Barcelona, Spain) and formic acid was from Amresco (Solon, OH, USA). Phenomenex (Torrance, CA, USA) Strata X SPE cartridges (500 mg/6 mL, surface area 800 m^2 g^{-1}, particle size 33 μm, average pore size 85 Å) were used to extract analytes from water samples.

2.2. Sampling

Samples were collected from the influent and effluent of 3 different WWTPs in Valencia (Pinedo I, Pinedo II and Quart-Benàger), receiving wastewater from Valencia and other small surrounding towns. The catchment area of Pinedo I and Pinedo II consists of 351,000 and 943,000 inhabitants, respectively, while the catchment area of Quart-Ben`ager consists of 215,000 inhabitants. The main characteristics of each WWTP are summarized in Table 5.1. Sampling campaigns were carried out in 3 years, 2011 (March 9th to 15th), 2012 (April 17th to May 1st) and 2013 (March 6th to 12th).

Samples (24 h composite) were collected using the operational equipment of the WWTPs in a time-proportional manner at 60 min time intervals. Sampling strategies for wastewater were already examined by Ort et al. (2010) and evaluated by Castiglioni et al. (2013). Composite samples instead of grab samples were selected in this study to get the maximum representativeness. A daily grab sample would never be representative because the concentration of the analytes varies along the day. Although some compounds can be significantly degraded (>15%, after 12 h) in composite samples (Baker et al., 2012), the analytes selected in this study for back-calculations AMP, MAMP, BEGC, KET and THCOOH are generally stable (Baker et al., 2012; Castiglioni et al., 2013). The sampling interval (60 min) was fixed by the WWTPs. According to Ort et al. (2010) and Castiglioni et al. (2013), considering population size, uncertainty is lower than 5% using this time interval.

All samples were stored in polyethylene terephthalate (PET) bottles. Once the samples arrived at the laboratory they were immediately frozen at −20 °C until analysis to prevent degradation of the illicit drugs.

2.3. UHPLC-MS/MS

2.3.1. UHPLC-QqQ-MS/MS

Chromatographic separation was performed using an Agilent 1260 UHPLC (Agilent, Waldbronn, Germany). The separation was carried out with a Phenomenex Kinetex C18 (1.7 μm, 100 A, 50 × 2.10 mm) at temperature of 30 °C and constant flow rate of 0.2 mL min^{-1}. The mobile phase was eluent A (formic acid 0.1% in water) and eluent B (formic acid 0.1% in methanol). The gradient elution was started at 10% B maintained for 5 min, then increased linearly to 95% B in 12 min and remained constant at 95% B up to 25 min, then it returns to the initial conditions with an equilibrium time of 15 min. The injection volume was 5 μL. The injection was long to use a flow compatible with MS detection. The UHPLC was coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization source working in the positive ionization (ESI^+) mode, 300 °C gas temperature, 11 L min^{-1} gas flow and 25 psi nebulizer. The optimal quantification and confirmation transitions as well as the specific MS parameters were optimized for each compound (Table S5.1).

2.3.2. UHPLC-QqTOF-MS/MS

The chromatography was performed on an Agilent 1260 Infinity using the same conditions as in the previous section. The UHPLC system was coupled to a hybrid quadrupole time-of-flight ABSciex Triple TOF™ 5600 (Framingham, MA, USA). The MS acquisition was performed in positive ionization using information-dependent acquisition (IDA) that consist of two experiments: the survey scan type that was a full scan mass spectrum between m/z 100–950 and the information dependent scan that was product ion mass spectrum of the selected precursor: ion spray voltage, 5500 V; declustering potential (DP) 80 V; collision energy (CE) 10 V; temperature 450 °C with curtain gas (CUR) 30 (arbitrary units); ion source gas 1 (GS1) 35 and ion source gas 2 (GS2) 35. IDA MS/MS was performed using the following criteria: ions that exceeded 100 cps, ion tolerance 50 mDa, and collision energy fixed at 45 V.

Table 5.1
Description of the different WWTPs.

	Pinedo I	Pinedo II	Quart-Benàger
Population served (hl)	351,000	943,000	215,000
Municipalities	Valencia	Valencia and surrounding towns	Valencia and surrounding towns
Treated wastewaters	100% Urban	100% Urban	40% Urban/60% industrial
Treated technology			
Primary	Settlement/physicochemical	Settlement/physicochemical	Settlement/physicochemical
Secondary	Activated sludge	Denitrification by activated sludge	Activated sludge/phosphorous removal
Tertiary	Coagulation/flocculation/filtration	Coagulation/flocculation/filtration	Coagulation/flocculation
Influent characteristics			
Flow (m^3/day)	117,211 ± 2633	244,817 ± 48,565	34,888 ± 4706
T °C	17.2	17.3	18.4
pH	7.7	7.6	7.8
BOD_5 (mg L^{-1})	223.1	264.2	367.1
COD (mg L^{-1})	396.3	473.4	625.4
N (mg L^{-1})	36.4	37.0	55.7
P (mg L^{-1})	4.9	4.7	7.3
NH_4 (mg L^{-1})	31.7	25.2	38.8
Effluent characteristics[a]			
T °C	15.3	14.6	18.0
pH	7.6	7.4	7.5
BOD_5 (mg L^{-1})	9.7	2.4	5.0
COD (mg L^{-1})	40.8	15.8	30.6
N (mg L^{-1})	32.4	6.7	9.3
P (mg L^{-1})	2.4	0.3	0.7
NH_4 (mg L^{-1})	17.0	0.2	3.9

T: temperature; BOD_5: biochemical oxygen demand; COD: chemical oxygen demand; N: nitrogen; P: phosphorus; NH_4: ammonium.
[a] The effluent flow is the same as influent flow.

Data acquisition and processing was carried out using software Analyst, Peak View 1.0 with the application XIC manager (to search the analytes against a database) and MultiQuant 2.0. The XIC manager displayed mass, mass error (PPM), retention time isotopic pattern and library (MS and MS/MS) identification of each peak detected.

2.4. Sample preparation

Samples (250 mL) were vacuum filtered with 0.45 μm retention capacity to remove solid particles before the solid-phase extraction (SPE) procedure. ISs were added before the filtering step and equilibrated for 15 min to allow the adsorption to suspended particulate matter. This step can be a source of uncertainty because it was unknown whether the equilibrium was reached or the absorption kinetics were comparable since IS was not at the same concentration that the analyte. However, this step was not validated because recently, Baker et al. (2012) demonstrated that this partitioning was less than 5% for analytes including COC, BECG, AMP, MAMP and MDMA. This indicated that it is not an important factor affecting the uncertainty of the analysis. Illicit drugs were extracted using Strata-X cartridges, previously conditioned with 6 mL methanol and 6 mL of Milli-Q water. Samples without pH adjustment were passed through the cartridges under vacuum, at a flow rate of 10 mL min^{-1}. After that, the cartridges were washed with 6 mL of Milli-Q water and dried under vacuum for 15 min. Analytes were eluted with 6 mL of methanol, evaporated to dryness and dissolved in 1 mL of water-methanol (9:1 v/v).

2.5. Estimation of collective illicit drug use

Illicit drugs consumptions were back-calculated from the measured daily concentration of the drug target according to the following equation (Eq. (5.1)) (Zuccato et al., 2008):

$$Q\left(\mathrm{mg}\Big/\mathrm{day}\right)\Big/1000\mathrm{inh.} = C\left[\frac{\mathrm{ng}}{\mathrm{L}}\right]*10^{-6}\left[\frac{\mathrm{mg}}{\mathrm{ng}}\right]*ST*FR\left[\frac{\mathrm{L}}{\mathrm{day}}\right]*\frac{1000}{Ninh}*\frac{100*MR}{Mex} \quad (5.1)$$

where *Q* is the consumption drug, *C* is the concentration, *ST* is the correction factor for stability (Van Nuijs et al., 2011c), *FR* is daily flow rate of each WWTP, *Ninh* is the number of inhabitants, *MR* is the molar ratio between the parent drug and the drug residue, *Mex* is the percentage of metabolic excretion (for detailed values see Table S5.2).

2.6. Statistical analysis

All statistical analyses were performed using SPSS for Windows 19.0 (SPSS, Inc.). Normal distribution was checked by Kolmogorov–Smirnov and Shapiro–Wilk tests for all variables. Statistical significant differences of median were judged by one-way analysis of variance (ANOVA) and least significant differences calculations at a 5% significant level.

2.7. Risk assessment of drugs of abuse in water

The risk assessment for aquatic organisms is performed calculating the risk quotient (RQ) Eq. (5.2) (Aldenberg and Slob, 1993):

$$\mathrm{RQ} = \frac{\mathrm{MEC}}{\mathrm{PNEC}} \quad (5.2)$$

where MEC is the measured environmental concentration and PNEC is predicted no-effect concentration. PNEC was calculated from EC50 and LC50, which were estimated by ECOSAR program (v 1.11) at 3 different trophic levels of the ecosystem, algae, daphnids and fish (Table S5.3). There are different risk levels interpreting the RQ in risk assessment ("low" from 0.01 to 0.1; "medium" from 0.1 to 1, and "high" >1)(Hernando et al., 2006).

3. Results and discussion

3.1. Validation of analytical procedures

Limit of quantification (LOQ), recovery, precision and matrix effect are outlined in Table 5.2. The linearity was also evaluated by injecting in triplicate 7 concentration levels from 1 to 1000 ng L^{-1} using IS calibration to avoid errors due to matrix effect. Calibration curves were linear with correlation coefficient (R^2) > 0.99 for all compounds (Table S5.4). The chromatogram corresponding to the calibration standard at 100 ng L^{-1} is shown in Fig. 5.1A. The matrix effects were compensated by using deuterated ISs in the wastewaters (Berset et al., 2010). The combination of such matrix effect and extraction efficiency was estimated from the IS, which were spiked at the same concentration into all samples.

Table 5.2
Limits of quantification (LOQ), recovery, inter-day and intra-day precision, and matrix effects of the SPE and UHPLC-QqQ-MS/MS method.

Compound	IS	LOQ (ng L^{-1})		Recovery (%; mean ± RSD) (n = 5)				Precision (%RSD) intra-day/inter-day (n = 5)				Matrix effects (%; mean ± RSD) (n = 5)							
				Ultrapure water			Influent wastewater			Ultrapure water			Influent wastewater			Influent wastewater (ISs 25 ng L^{-1})			Effluent wastewater (ISs 25 ng L^{-1})	
		LOQ	S/N (S/N)	At LOQ	500 ng L^{-1}	100 ng L^{-1}	500 ng L^{-1}	At LOQ	500 ng L^{-1}	100 ng L^{-1}	500 ng L^{-1}	PI	PII	QB	PI	PII	QB
6-ACMOR	6-ACMOR-d_3	1	6.6 (4.4)	115 ± 16	133 ± 20	75 ± 19	80 ± 17	18.6/9.5	13.7/8.4	14.8/10.1	10.3/11.3	78.4 ± 15.4	127.5 ± 17.6	106.7 ± 19.3	79.2 ± 18.5	81.7 ± 7.3	56.2 ± 6.5
AMP	AMP-d_5	1	11.0 (2.4)	87 ± 2	93 ± 4	76 ± 8	94 ± 5	15.6/9.9	12.9/8.3	12.6/8.4	16.1/6.2	53.1 ± 10.3	85.6 ± 20.9	70.4 ± 19.5	75.5 ± 1.9	61.6 ± 12.5	57.9 ± 1.4
BECG	BECG-d_3	1	14.8 (3.2)	91 ± 8	101 ± 9	102 ± 20	92 ± 9	10.6/9.1	10.3/7.9	12.1/7.1	12.6/5.9	37.1 ± 6.4	62.6 ± 16.7	37.3 ± 20.0	117.2 ± 7.9	108.0 ± 11.8	91.6 ± 1.8
COC	COC-d_3	1	8.6 (7.1)	99 ± 18	106 ± 20	114 ± 19	76 ± 20	17.2/6.7	10.2/8.7	15.2/2.9	12.1/6.9	127.5 ± 1.0	245.1 ± 19.1	38.6 ± 18.2	16 ± 2.1	93.2 ± 4.9	16.0 ± 7.0
KET	KET-d_4	1	6.2 (4.1)	89 ± 14	93 ± 19	90 ± 12	96 ± 7	11.9/7.8	12.9/6.8	15.4/1.0	14.9/7.0	72.1 ± 7.6	128.4 ± 17.9	117.7 ± 17.9	107.3 ± 7.6	109.1 ± 2.1	97.7 ± 15.5
MAMP	MAMP-d_5	1	14.0 (11.4)	85 ± 6	90 ± 7	84 ± 4	92 ± 2	15.6/15.3	15.0/12.3	16.0/19.2	9.2/111	68.2 ± 11.1	112.9 ± 18.7	92.6 ± 17.3	96.3 ± 2.8	85.3 ±19.3	60.6 ± 2.7
MDMA	MDMA-d_5	1	14.6 (4.5)	86 ± 4	90 ± 4	86 ± 13	101 ± 6	11.8/12.5	9.1/14.3	11.9/7.8	17.2/14.5	70.4 ± 11.6	108.1 ± 19.1	66.0 ± 14.9	103.6 ± 7.4	102.1 ± 2.0	96.3 ± 3.7
THC-COOH	THC-COOH-d_3	1	9.4 (5.5)	65 ± 6	68 ± 6	66 ± 7	100 ± 20	18.0/21.9	19.1/21.2	20.0/19.9	18.0/21.1	15.9 ± 13.1	14.8 ± 16.9	4.2 ± 12.2	43.6 ± 1.7	30.4 ± 10.9	48.0 ± 7.3

IS: deuterated internal standard; R^2: coefficient of determination; LOQ: limit of quantification; S/N: signal/noise ratio of quantifier transition; (S/N):signal/noise ratio of qualifier transition; RSD: % relative standard deviation; n: number of replicates; PI: Wastewater Treatment Plant of Pinedo I; PII: Wastewater Treatment Plant of Pinedo II; QB: Wastewater Treatment Plant of Quart-Benàger.

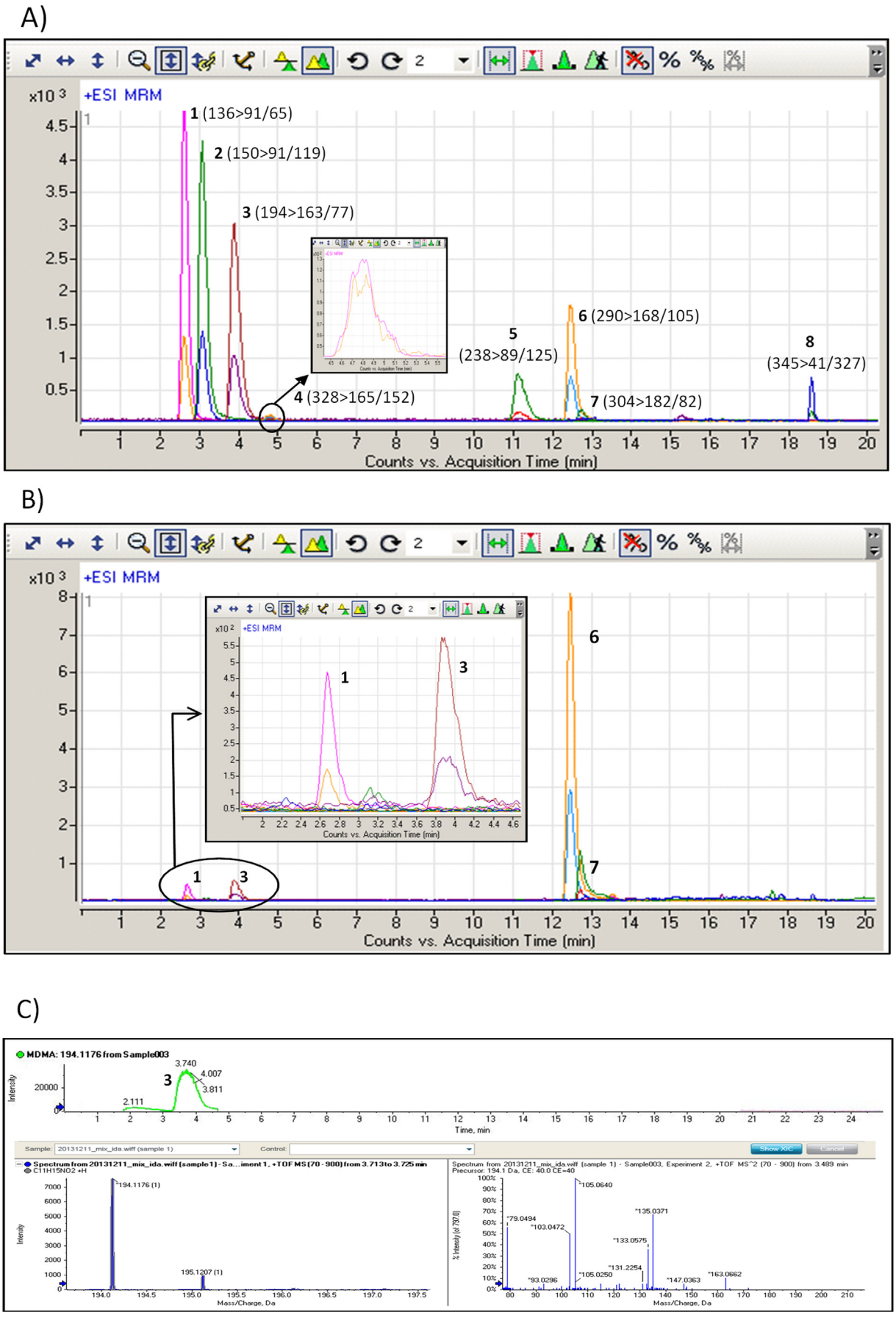

Fig. 5.1. Chromatogram of 100 ng L^{-1} of the calibration standard solution (product ion > quantifier transition/qualifier transition) (A). Chromatogram of a wastewater influent sample showing the compounds detected (B). Full scan accurate mass precursor and product ion spectrum of MDMA were identified in influent wastewater sample with UHPLC-QqTOF-MS/MS (C). (1) AMP, (2) MAMP, (3) MDMA, (4) ACMOR, (5) KET, (6) BECG, (7) COC, (8) THC-COOH.

Table 5.3
Frequency of detection in the influent of WWTPs of illicit drugs and metabolites (%) and levels quantified (mean and range).

	Influent Pinedo I			Influent Pinedo II			Influent Quart-Benàger		
	Freq (%)	Loads (g day^{-1})		Freq (%)	Loads (g day^{-1})		Freq (%)	Loads (g day^{-1})	
		Mean	Range		Mean	Range		Mean	Range
2013									
6-ACMOR	n.d.	<LOQ		n.d.	<LOQ		n.d.	<LOQ	
AMP	100	6.5	3.9–10.0	100	6.7	4.6–9.3	100	2.2	0.5–3.6
BECG	100	156.7	99.6–221.3	100	310.4	218.1–408.2	100	90.3	32.7–160.9
COC	100	86.1	46.1–216.3	100	270.0	107.9–633.4	100	43.4	11.3–75.7
KET	100	2.5	0.7–11.9	100	12.8	1.4–43.9	100	0.6	0.4–1.3
MAMP	100	0.5	0.3–0.8	100	0.9	0.7–1.2	100	0.1	0.1–0.2
MDMA	100	4.5	1.0–11.5	100	5.2	1.4–12.5	100	1.3	0.2–3.3
THC-COOH	100	25.6	15.9–34.8	100	53.5	44.5–88.7	100	12.9	5.2–19.5
2012									
6-ACMOR	n.d.	<LOQ		6.7	4.0	4.0–4.0	n.d.	<LOQ	
AMP	100	10.3	6.4–12.4	100	18.7	13.7–29.2	100	0.1	0.06–0.2
BECG	100	195.6	108.9–307.4	100	426.2	262.3–679.2	100	134.0	74.6–209.6
COC	100	34.8	20.9–59.8	100	52.1	40.8–70.2	100	0.7	0.4–0.8
KET	100	1.9	1.0–2.7	100	4.2	2.4–5.6	100	0.04	<LOQ–0.05
MAMP	100	5.1	3.2–6.3	100	11.1	9.4–15.1	100	0.07	0.04–0.09
MDMA	100	2.1	0.9–4.2	100	4.7	2.9–7.9	100	0.03	<LOQ –0.04
THC-COOH	100	9.7	6.2–17.3	100	8.9	4.8–16.7	100	2.2	0.9–3.7
2011									
6-ACMOR	n.d.	<LOQ		n.d.	<LOQ		n.d.	<LOQ	
AMP	100	15.0	12.3–21.4	100	22.5	18.0–30.6	100	3.2	1.8–4.7
BECG	100	257.7	167.0–400.2	100	476.7	355.5–965.9	100	118.6	75.4–184.0
COC	100	57.0	34.7–99.1	100	70.6	49.8–122.8	100	11.7	7.2–17.3
KET	n.a.			n.a.			n.a.		
MAMP	100	7.3	6.3–9.1	100	12.8	10.7–15.7	100	1.7	1.2–2.2
MDMA	100	2.2	1.1–5.0	100	3.8	2.5–8.3	100	0.4	0.2–0.7
THC-COOH	100	10.4	2.8–18.0	100	12.7	5.1–26.2	100	2.6	0.5–4.5

Freq: frequency; <LOQ: below limit of quantification; n.d.: not detected; n.a.: not analyzed.

LOQs of the method were determined by analysis of spiked distilled water as the concentration of analyte giving a signal-to-noise ratios (S/N). Values of 1 ng L^{-1} were practically considered for all the analytes since LOQs could be worse in wastewater due to the signal suppression along with the higher background noise (S/N values at this concentration are also in Table 5.2). Extraction recoveries were evaluated in quintuplicated by spiking aliquots of deionized water with the analytes at the LOQ and at 500 ng L^{-1} of each analyte and of influent wastewater at 100 and 500 ng L^{-1}. In the latter, as all analytes except 6-ACMOR were in the sample, five aliquot of a sample with low concentrations were spiked at each concentration and other five aliquots of this sample were measured without analytes spike. Then, the spiked samples, after non-spiked sample signal subtraction (non-spiked samples concentrations were COC 400 ng L^{-1}, BECG 800 ng L^{-1}, MDMA 8 ng L^{-1}, AMP 20 ng L^{-1}, MAMP 3 ng L^{-1}, THCOOH 220 ng L^{-1} and KET 6 ng L^{-1}) was compared to the calibration curve. Recoveries were 68–133% in deionized water and 76–101% in influent wastewater at 500 ng L^{-1}. The results show that recoveries of COC, BECG and 6-ACMOR were better in deionized water than in influent wastewater. For AMP, KET, MAMP, MDMA and THC-COOH the recoveries were better in influent. The lower recovery for cocainics in wastewaters could be due to the sample complexity whereas the high recovery for the other compounds could be attributable to an enhancement of the extraction efficiency due to their higher ionic strength (Baker and Kasprzyk-Hordern, 2013; Boleda et al., 2009). The repeatability and reproducibility between days of five samples spiked at 100 and 500 ng L^{-1} in both types of matrix, expressed as % relative standard deviation (%RSD), were lower than 20% and 23%, respectively (Table 5.2).

Fig. 5.1B illustrates the chromatogram corresponding to a wastewater influent in which AMP, MDMA, COC and BECG were detected at concentrations of 110.0, 158.4, 3192.5 and 7752.5 ng L^{-1} respectively. The insert magnifies the AMP and MDMA peaks to shows both transitions.

The correct identification of target compounds was ensured by UHPLC-QqTOF-MS/MS. Fig. 5.1C illustrates the identification of MDMA by accurate MS and MS/MS against a user-built library. Accurate mass spectra of all samples were acquired in the *m/z* range 100–950, which achieves confirmation of drugs of abuse identities by searching these compounds against a MS/MS library. Purity score values higher than 75% were always obtained in MS/MS identifica-tion against the library and LOQ were equal those obtained by QqQ.

3.2. Occurrence of illicit drugs and metabolites in wastewaters

Table 5.3 summarizes corresponding loads (g day^{-1}; mean and range values) and frequency of detection of individual analytes in the 3 WWTPs according to the year. The same information but in concentration (ng L^{-1}), is listed in Table S5.5. 6-ACMOR (minor but exclusive metabolite of heroin) was only detected in the influent of Pinedo II on Wednesday 25th 2012 at a concentration of 16.6 ng L^{-1} (mean load 4.0 g day^{-1}), so, it was not considered for the following data analysis. Not many studies use 6-ACMOR for estimating consumption of heroin because its low excretion rate (1.3%) and rapid degradation in wastewaters (Postigo et al., 2010; Boleda et al., 2009; Huerta-Fontela et al., 2008; Zuccato et al., 2008). Its absence in WWTPs was also noted by several authors (Boleda et al., 2009; Van Nuijs et al., 2009a). Others have reported low but measurable concentrations of 6-ACMOR (Terzic et al., 2010). This can be

explained by the different prevalence of heroin consumption between countries.

Concentrations of COC and its main metabolite BECG ranged from 110.3 to 3429.7 ng L^{-1} (mean load ranged from 0.7 to 270.0 g day^{-1}) and from 870.9 to 7752.5 ng L^{-1} (mean load ranged from 90.3 to 476.7 g day^{-1}), respectively. These data are in agreement with those reported in Europe (Kasprzyk-Hordern et al., 2009b; Martínez Bueno et al., 2011; Postigo et al., 2008). The highest concentration of cocainic compounds (COC and BECG) was observed in Quart-Benàger.

Amphetamine-like stimulants ranging from 1.7 to 110.0 ng L^{-1} (mean load ranged from 0.1 to 22.5 g day^{-1}) for AMP, from 1.2 to 69.1 ng L^{-1} (mean load ranged from 0.07 to 12.8 g day^{-1}) for MAMP and from <LOQ to 159.1 ng L^{-1} (mean load ranged from 0.03 to 5.2 g day^{-1}) for MDMA, in good agreement with other results (Berset et al., 2010; Castiglioni et al., 2006; González-Mariño et al., 2011; Postigo et al., 2010; Van Nuijs et al., 2011b). The highest concentration levels of MAMP were detected in Pinedo I, whereas those of AMP and MDMA were in Quart-Benàger.

THC-COOH ranging from 18.8 to 940.2 ng L^{-1} (mean load ranged from 2.2 to 53.5 g day^{-1}). In Spain, THC-COOH was detected in similar concentrations by González-Mariño et al. (2011). THC-COOH was found in highest concentration in 2013 and 2011 in Quart-Benàger. In 2012 the highest concentration detected of THC-COOH was in Pinedo I.

Regarding KET, concentrations were between < LOQ and 131.8 ng L^{-1} (mean load ranged from 0.04 to 12.8 g day^{-1}). These results agree to those obtained by Huerta-Fontela et al. (2008). In 2012 and 2013 the highest concentration detected of this compound was in Pinedo I and Pinedo II respectively. KET was not analyzed in 2011 campaign.

3.3. Consumption estimation

Average consumption rate calculated at the 3 selected WWTPs in 2011, 2012 and 2013 of each drug of abuse expressed as mg day^{-1} 1000 $inhabitants^{-1}$ is shown in Fig. 5.2. The most important differences between the 3 WWTP is the population they serve. Pinedo I only serves the population of Valencia, which is the capital city of the Valencia Community and the third largest city in Spain, with many leisure places and an intensive night live. Pinedo II and Quart-Benàger cover, in addition to a part of Valencia, several province towns. Furthermore, the latter also covers an important industrial belt that surrounds Valencia.

Most consumed drugs were COC and cannabis. The estimated COC consumption in 2011 was 1641.3, 1181.7, 1332.6 mg day^{-1} 1000 $inhabitants^{-1}$ in Pinedo I, Pinedo II and Quart-Benàger, respectively. In 2012 the consumption of COC decreased in comparison to 2011 in Pinedo I and II. The trend through low COC consumption was confirmed in 2013 for the 3 WWTPs. The highest consumption of COC was detected in Pinedo I in 2011 and 2013, which could be related with the urban habits of Valencia. COC use in the 3 WWTP is similar to that observed in Catalonia (Spain) and London (United Kingdom) (Huerta-Fontela et al., 2008; Zuccato et al., 2008) and higher than other territories as Milan (Italy), Lugano (Switzerland), Brussels (Belgium) and South Wales (United Kingdom) (Kasprzyk-Hordern et al., 2009a; Van Nuijs et al., 2011c; Zuccato et al., 2008). Average values in the 3 years of COC consumption in doses were 13.7, 11.1 and 13.3 doses day^{-1} 1000 $inhabitants^{-1}$ of COC in Pinedo I, Pinedo II and Quart-Benàger, respectively (standard dose 100 mg) (Terzic et al., 2010; Zuccato et al., 2008).

Cannabis consumption was always high in Pinedo I (4034.4, 4163.2 and 12,422.5 mg day^{-1} 1000 $inhabitants^{-1}$ in 2011, 2012 and 2013 respectively) and underwent a large increase in 2013 in the 3 WWTPs. The estimated consumption in 2011 and 2012 in Pinedo II (1935.0 and 1579.6 mg day^{-1} 1000 $inhabitants^{-1}$ respectively) and Quart-Benàger (1880.0 and 1743.4 mg day^{-1} 1000 $inhabitants^{-1}$ respectively) is lower than in Zagreb (Croatia), Catalonia (Spain), Milan (Italy), Lugano (Switzerland) and London (United Kingdom) (Boleda et al., 2009; Terzic et al., 2010; Zuccato et al., 2008). The estimated consumption in 2013 in Pinedo I, Pinedo II and Quart-Benàger was much higher than in the cities mentioned above (12,422.5, 10,591.8 and 9419.8 mg day^{-1} 1000 $inhabitants^{-1}$ respectively). Equivalent averages doses for Pinedo I, Pinedo II and Quart-Benàger were 55.0, 37.6 and 34.8 doses day^{-1} 1000 $inhabitants^{-1}$, respectively (doses of 125 mg) (Terzic et al., 2010). The temporal trend from 2011 to 2013 indicates a decrease of COC consumption and an increase in cannabis. The same trend has been established through European cities as reported in recent study (Ort et al., 2014).

The recreational use of KET is difficult to ascertain due its use as an anesthetic in hospitals and as a veterinary medicine too. The highest estimated consumption was in Pinedo I in 2012 (240.4 mg day^{-1} per 1000 $inhabitants^{-1}$) and in Pinedo II in 2013 (694.8 mg day^{-1} per 1000 $inhabitants^{-1}$). The values detected in the 3 WWTPs increased in 2013. The increase of Pinedo II and Quart-Benàger (115.0 mg day^{-1} per 1000 $inhabitants^{-1}$) were higher than in Pinedo I (278.6 mg day^{-1} per 1000 $inhabitants^{-1}$). This could signify an increase in the consumption of KET as drug of abuse in front of its use as pharmaceutical.

Pinedo I was the WWTP where highest consumption of AMP was detected in the 3 campaigns (135.5, 97.5 and 70.8 mg day^{-1} 1000 $inhabitants^{-1}$ in 2011, 2012 and 2013 respectively). These values were similar to results reported in Catalonia (Spain), London (United Kingdom), Brussels (Belgium) (Huerta-Fontela et al., 2008; Van Nuijs et al., 2011c; Zuccato et al., 2008). The trend of consumption of AMP during the 3 years was decreasing up to 2013, when the lowest value was detected in Pinedo II (29.9 mg day^{-1} 1000 $inhabitants^{-1}$), although the lowest value detected in the 3 campaigns was in Quart-Benàger in 2012 (2.2 mg day^{-1} per 1000 $inhabitants^{-1}$). Doses usually applied are 50 mg for AMP (Terzic et al., 2010), so the average doses corresponding to Pinedo I, Pinedo II and Quart-Benàger were 2.03, 1.21 and <1 doses day^{-1} 1000 $inhabitants^{-1}$.

Equally, the MAMP consumption decreased from 2011 to 2013, from 45.5 to 3.9 in Pinedo I, from 30.3 to 2.8 in Pinedo II and from 17.5 to 1.3 mg day^{-1} 1000 $inhabitants^{-1}$ in Quart-Benàger. As in the case of the AMP the highest values were detected in Pinedo I and the lowest in Quart Benàger. The values of 2013 were close to the values detected in Catalonia (Spain) (Postigo et al., 2010) The consumption in Valencia in 2011 and 2012 (33.8 and 30.4 mg day^{-1} per 1000 inhtabitants^{-1} in Pinedo I and Pinedo II respectively) was higher than those reported for Milan (Italy) or London (United Kingdom) (Zuccato et al., 2008). The average estimated consumption of MAMP (each dose ca. 40 mg) (Postigo et al., 2010) was <1 doses day^{-1} 1000 $inhabitants^{-1}$ respectively in the 3WWTP.

Regarding MDMA the highest estimated consumption was in Pinedo I (9.2, 9.0 and 21.9 mg day^{-1} 1000 $inhabitants^{-1}$ in 2011, 2012 and 2013 respectively). The lowest values were in Quart-Benàger (2.9, 0.2 and 10.1 mg day^{-1} 1000 $inhabitants^{-1}$ in 2011, 2012 and 2013 respectively). These values are in agreement with those detected in Lugano (Switzerland), Milan (Italy), London (United Kingdom), Zagreb (Croatia) (Terzic et al., 2010). In contrast to what happens with AMP and MAMP, the consumption of MDMA increase in 2013 in the 3 WWTPs. The corresponding average values in terms of dose in Pinedo I, Pinedo II and Quart-Benàger were <1 doses day^{-1} 1000 $inhabitants^{-1}$ in the 3 WWTPs, if the dose of MDMA corresponds to 100 mg for MDMA (Postigo et al., 2010).

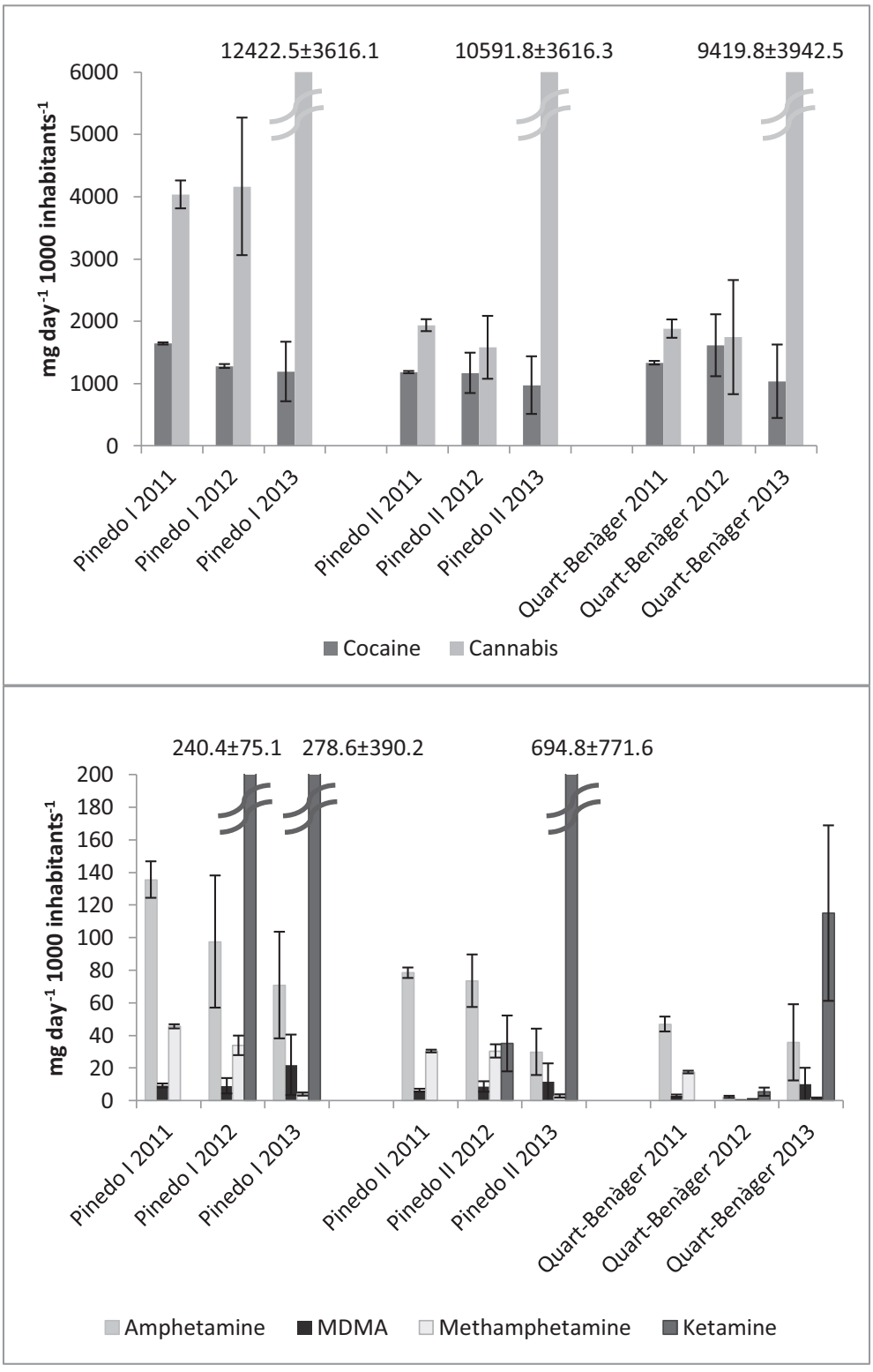

Fig. 5.2. Average consumption at the community level in the city of Valencia at the 3 WWTPs in 2011 ($n = 7$), 2012 ($n = 15$) and 2013 ($n = 7$) of each drug of abuse.

ANOVA analysis was performed in order to investigate whether the differences in consumption among the 3 WWTPs were statistically significant. According to the data obtained particularly for cannabis and COC, Pinedo I shows a high consumption rates that could be because this WWTP only treats water from Valencia, which has a lot of social and night life. In fact, much more than the other surrounding towns, which are just dormitory towns and/or industrial areas. However, differences observed between the 3 WWTP were not statistically significant.

3.4. Daily consumption variations

Daily consumption of the drugs of abuse in the 3 campaigns in the influent of Pinedo I, Pinedo II and Quart-Benàger are illustrated

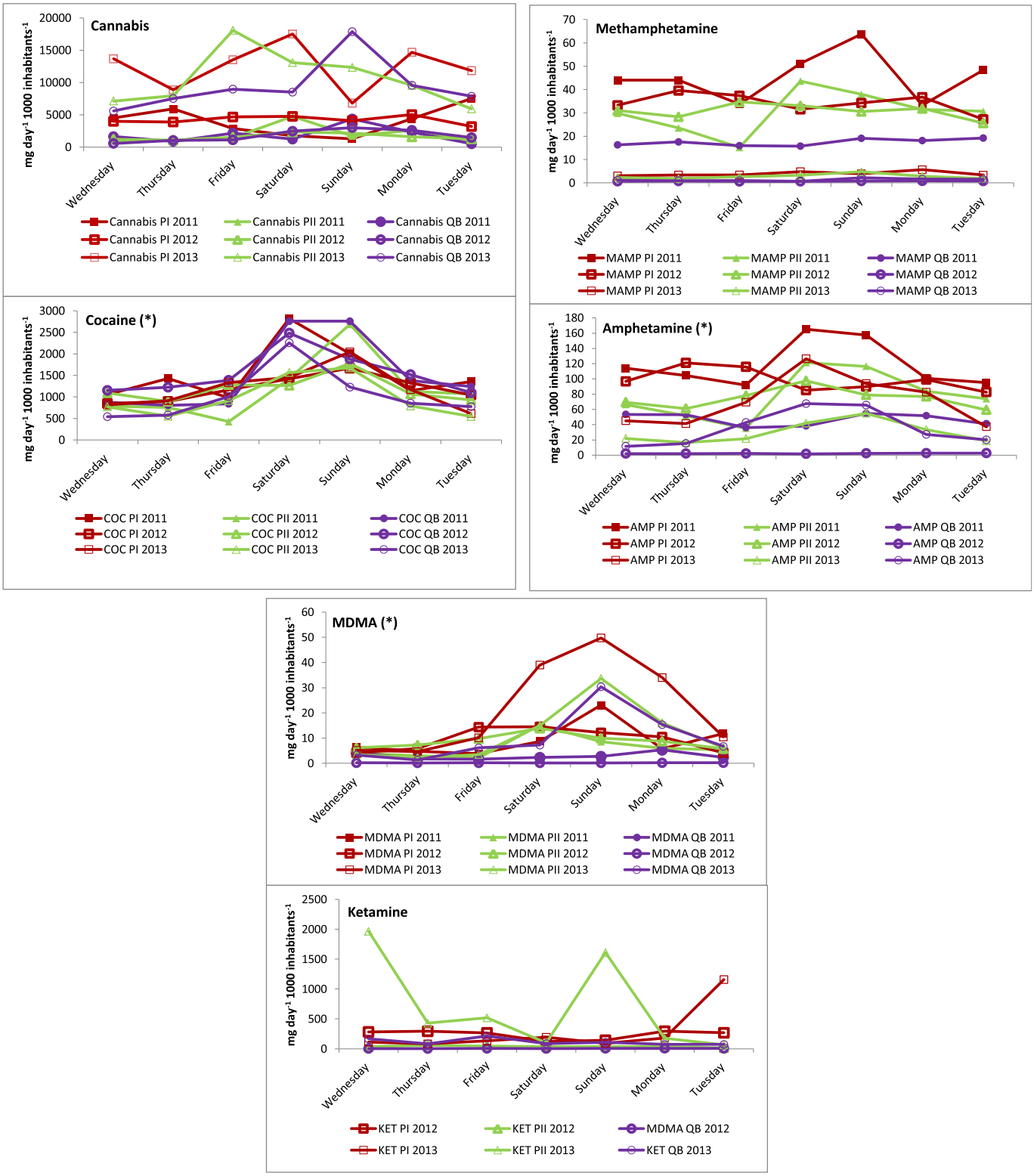

Fig. 5.3. Daily consumption of the investigated drugs of abuse in the 3 campaigns in the influent from the WWTPs in the city of Valencia. (*) Differences between weekdays (Monday to Thursday) and weekend days (Friday to Sunday) were statistically significant for these drugs of abuse.

in Fig.5.3. COC and MDMA show a clear recreational trend. A statistical study was carried out to establish differences among weekdays (Monday to Thursday) and weekend days (Friday to Sunday). These differences were statistically significant for COC, MDMA and AMP ($p < 0.05$), but not for KET, MAMP and cannabis. These trends are consistent with those reported in other countries (Bijlsma et al., 2014; Huerta-Fontela et al., 2008; Terzic et al., 2010).

The measured COC/BECG ratios expected when considering the metabolism and excretion pattern of COC ranged from 0.3 to 0.7 (Postigo et al., 2010; Van Nuijs et al., 2009a) depending on

environmental conditions such as temperature or residence time. Data obtained in the present study are outlined in the supplementary information Fig. S5.1. In 2011 and 2012, mean ratio obtained is within the range (between 0.2 and 0.5). However, 2 influent samples collected in 2013 from Quart-Ben`ager and Pinedo II showed unexpectedly high COC/BECG ratios (2.0 on Friday and 1.6 on Sunday, respectively). Furthermore, on Saturday, the 3 WWTPs present a ratio near 1. All these findings indicate disposal of non-consumed COC into the sewage system (BECG constant equal in-crease in COC). On Thursday, according to the newspapers (Hortanoticias.com, 2013) the national Spanish police dismantled a COC sale point located at a home in Manises (covered by Quart-Ben`ager). That could also have an influence in other parts of the city by several police searches. The non-regular consumption of COC was also noted by Bijlsma et al. (2012). Several approaches as chiral analysis can help in these identifications as already demonstrated by Baker et al. (2012).

3.5. Removal efficiency

Removal efficiency was calculated from the concentrations measured in the influent and effluent wastewater as an average removal of the drugs of abuse in the investigated WWTPS. Fig. 5.4 shows the comparison of the mean removal of illicit drugs and metabolites (for 2012 and 2013). The compounds with the highest removals (never detected in the effluent independently of the WWTP under study) were AMP, MAMP, THC-COOH and COC, which is in agreement with other reports (Huerta-Fontela et al., 2008; Postigo et al., 2010). BECG removal efficiency varied among WWTPs, the values ranged from 93.4% in Quart-Benàger to 98.5% in Pinedo II, which is also in agreement with other reports (Huerta-Fontela et al., 2008; Postigo et al., 2010).

MDMA removal efficiency is variable depending on the WWTPs ranging from 32 to 57%. It was higher in Pinedo II (53.1%–56.8%) followed by Pinedo I (55.9% in 2011and 51.6% in 2012) and Quart-Benàger (46.8% in 2012 and 32.3 in 2013) in agreement with (Huerta-Fontela et al., 2008). The only difference between the 3 WWTPs is the secondary treatment applied (see Table 5.1).

Negative removal values were found for KET (Fig. 5.4), which corroborate published studies establishing that KET is not susceptible to WWTP treatments (Huerta-Fontela, 2008; Baker and Kasprzyk-Hordern, 2013) and contradict other that detect lower concentrations in effluents than in influents (Postigo et al., 2010). Plausible justifications for these unfavorable or negative removal rates are residence times (<24 h), the deconjugation of metabolites and/or transformation products and desorption from particulate matter during wastewater treatment (Baker et al., 2012; Postigo et al., 2010).

Drugs of abuse detected in WWTP effluents (Table S5.6) in the <LOQ–755.0 ng L^{-1} range were BECG, KET and MDMA with the highest prevalence. On the contrary, AMP, COC, MAMP and THC-COOH were non-detected in the effluent of the WWTPs. Because of the treatment efficiency and as a mean value considering only the last year 2013, Pinedo I, discharges a total of 6.7 g day^{-1} of BECG, 1.9 g day^{-1} of KET and 5.8 g of MDMA. Similarly, Pinedo II releases 4.5 g day^{-1} of BECG, 2.9 g day^{-1} of KET and 5.6 g of MDMA and Quart-Ben`ager release 4.1 g day^{-1} of BECG, 0.5 g day^{-1} of KET and 1.0 g day^{-1} of MDMA (Table 5.4). These compounds are mainly discharged associated with the dissolved phase, since treatment eliminates most of the suspended solids that enter the WWTP.

3.6. Risk assessment of the presence of drugs of abuse in water

Ecotoxicological risk of drugs of abuse released by WWTPs effluent into the environment was evaluated in this study by calculating risk quotient (RQ) for MDMA, KET and BECG for the 3 trophic levels of the ecosystem, algae, daphnids and fish. The results were summarized in Table S5.7. According to the RQ

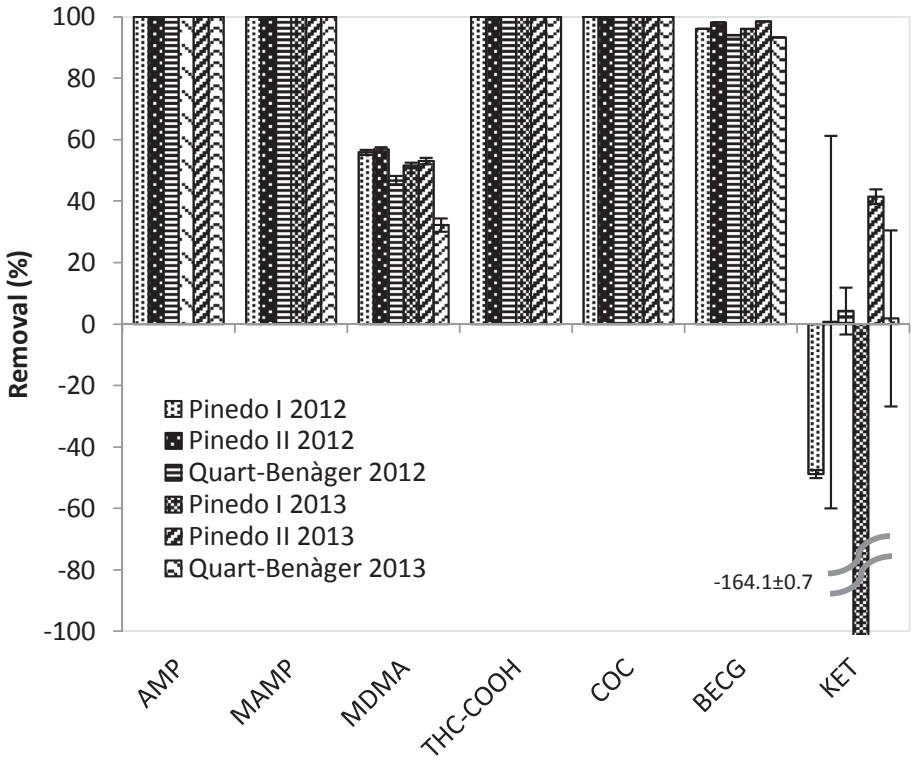

Fig. 5.4. Average removal of drugs of abuse and metabolites in selected WWTPs.

Table 5.4
Frequency of detection in the effluent of WWTPs of illicit drugs and metabolites (%) and levels quantified (mean and range).

	Effluent Pinedo I			Effluent Pinedo II			Effluent Quart-Benàger		
	Freq (%)	Loads (g day^{-1})		Freq (%)	Loads (g day^{-1})		Freq (%)	Loads (g day^{-1})	
		Mean	Range		Mean	Range		Mean	Range
2013									
6-ACMOR	n.d.			n.d.			n.d.		
AMP	n.d.			n.d.			n.d.		
BECG	100	6.7	0.5–13.1	100	4.5	0.7–13.9	100	4.1	2.2–7.9
COC	n.d.			n.d.			n.d.		
KET	100	1.9	0.8–2.8	100	2.9	1.1–4.4	100	0.5	0.4–0.8
MAMP	n.d.			14.3			n.d.		
MDMA	57.1	5.8	3.3–9.6	57.1	5.6	3.0–9.2	71.4	1	0.3–2
THC-COOH	n.d.			n.d			n.d.		
2012									
6-ACMOR	n.d.			n.d.			n.d.		
AMP	n.d.			n.d.			n.d.		
BECG	100	8.1	0.5–16.4	100	6.7	1.3–16.0	100	8.3	2.9–22.9
COC	n.d.			n.d.			n.d.		
KET	100	2.9	0.3–5.2	73.3	0.8	0.2–1.4	93.3	0.04	<LOQ–0.05
MAMP	n.d.			n.d.			n.d.		
MDMA	53.3	1.9	0.8–2.8	60	3.4	1.5–4.6	46.7	0.05	<LOQ–0.1
THC-COOH	n.d.			n.d.			n.d.		

Freq: frequency; n.d.: not detected.

classification ("low" from 0.01 to 0.1; "medium" from 0.1 to 1, and "high" >1) MDMA could pose medium risk because the highest value for RQ was 0.4 (daphnids). Regarding KET the highest value for RQ was 0.06 (algae) so could pose low risk. BECG could not pose risk, because its RQ was 0.0 in all scenarios. These data had been calculated in the effluent of the WWTPs, so this is a worst case scenario because effluents are diluted further in surface waters. Chronic exposure to illicit drugs can produce numerous and undetermined long-term toxicity effects, which cannot be deduced working with data on acute toxicity. These effects could induce metabolic or reproductive changes in non-target organism, toxicity by synergy or adding effects in aquatic organisms as well as other side toxic effects, not yet studied.

4. Conclusions

The analysis of sewage waters of the 3 main WWTPs that treat the wastewaters of Valencia provides valuable information of illicit drugs that are used in this area. Moreover, as both influent and effluent samples were analyzed in two consecutive years (2012 and 2013) for fifteen and seven days, respectively, removal efficiencies of the selected WWTPs were evaluated. These efficiencies were generally satisfactory (>95%) except for KET and MDMA. The RQ obtained from the measured concentrations in effluents (mean and maximum) and considering the PNEC values obtained using ECOSAR approach, no short-term environmental risk might be expected from the effluents of the WWTPs studied.

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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.2014.07.019.

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Supplementary Information

Occurrence and removal of drugs of abuse in Waste Water Treatment Plants of Valencia (Spain). A three-years survey.

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Table S5.1. Retention times (T_r), optimized MRM transition, collision energy (CE) used for UHPLC-MS/MS analysis and ratio between product ions of the target drugs of abuse.

	T_r (min)	MRM_1 transition (Quantifier)	CE (V)	MRM_2 transition (Qualifier)	CE (V)	MRM ratio (MRM_1/MRM_2)
6-ACMOR	4.77	328>165	41	328>152	81	0.5
6-ACMOR- d3	4.77	331>183	30			
AMP	2.63	136>91	13	136>65	41	3.4
AMP-d5	2.63	141>92	13	141>93	13	4.1
BECG	12.45	290>168	17	290>105	29	0.9
BECG-d3	12.45	293>171	17	293>105	33	1.7
COC	12.73	304>182	17	304>82	29	5.0
COC-d3	12.73	307>185	17	307>85	53	1.4
KET	11.22	238>89	69	238>125	25	5.0
KET-d4	11.22	242>129	25	242>92	73	1.2
MAMP	3.08	150>91	17	150>119	5	2.9
MAMP-d5	3.08	155>92	17	155>121	9	1.3
MDMA	3.90	194>163	10	194>77	50	2.0
MDMA-d5	3.90	199>165	9			
THC-COOH	18.48	345>41	73	345>327	9	7.0
THC-COOH-d3	18.48	348>193	33			

Table S5.2. Drug residues used to back calculate drug consumption, percentage of degradation, correction factors of stability and excretion pattern, average excretion rate and molar ratio.

	Drug residue	Degradation (%)	Correction factor for stability	Average excretion rate (%)	Molar ratio (MR)	Correction factor for excretion pattern
Heroin	6-ACMOR	30	1.43	1.3	1.13	86.8
Amphetamine	AMP	30	1.43	30	1	3.33
Cocaine	BECG	<5	1	45	1.05	2.33
Ketamine	KET	--	--	2.3	1	43.5
Methamphetamine	MAMP	<5	1	43	1	2.33
MDMA	MDMA	<5	1	65	1	1.54
Cannabis	THC-COOH	--	--	0.6	0.91	152

Sewage epidemiologic strategy described by Zuccato et al. (Zuccato et al., 2008) was employed to estimate illicit drug consumption through the influent drug concentration of the WWTPs investigated. Loads (g day^{-1}) for each compound were normalized for 1000 inhabitants using population estimate based in chemical oxygen demand (COD) for Pinedo I and II and in biochemical oxygen demand (BOD) for Quart-Benàger. Table 2 shows drugs residues used to back calculate drug consumption average excretion rates and correction factors. Cocaine is excreted by an average of 45% in the urine as BECG and only about 16% as the unchanged drug according different studies (Ambre, 1985;Baselt RC., 2008;Postigo et al., 2010;Zuccato et al., 2005). Due its concentration in wastewater was used to estimate the amounts of cocaine consumed by population. BECG loads (mg/day per 1000 inhabitants) at each sampling site calculated from multiplied by a factor 2.33 to estimate the load of parent cocaine. This factor was derived from the BEEG/cocaine molar mass ratio and the average moral fraction (45%) of a cocaine dose that is excreted as BEEG according to different studies (Ambre, 1985; Baselt RC., 2008). Amphetamine, Methamphetamine and MDMA calculation of consumption assumed that the proportion excreted unchanged in the urine and faeces after their ingestion was 30%. 43% and 65% respectively (Baselt RC., 2008). Molar mass ratio for these compounds is 1. So the factors used to convert the loads found in influent wastewaters into the drug amounts consumed are 3.3 for AMP 2.3 for MAMP and 1.5 for MDMA.

There are two metabolites to determine the consumption of heroin, morphine and 6-ACMOR. In this study, 6-ACMOR was measured due is a minor secondary metabolite. About 1.3% excretion rate of heroin but is exclusive of it (Postigo et al., 2008; Zuccato et al., 2005).

Cannabis has high metabolization of the main psychoactive compound THC and the excretion rates of secondary metabolites are very low (below 2%). Its estimate consumption is based on two major metabolites OH-THC and THCCOOH. THCCOOH was selected as cannabis consumption indicator to achieve comparable results with other studies (Boleda et al., 2009; Postigo et al., 2010).

Ketamine consumption was determinate as parent compound. Experiments evaluating the urinary excretion pattern of KET suggest that a KET dose is excreted as unchanged drug (2.3%). NK (1.6%) and conjugates of hydroxylated derivatives (> 80%) (Baselt RC., 2011; Kim et al., 2008).

Table S5.3. Lowest available EC50 values (mg L^{-1}) for algae, daphnids and fish and derived PNECs (mg L^{-1}).

Target Compound	EC50 Algae	LC 50 Daphnids	LC 50 Fish	AF	PNEC Algae	PNEC Daphnids	PNEC Fish
	mg L^{-1}	mg L^{-1}	mg L^{-1}		mg L^{-1}	mg L^{-1}	mg L^{-1}
6-ACMOR	6.17	1.84	2.68	1000	6.17E-03	1.84E-03	2.68E-03
AMP	3.8	4.36	37.6	1000	3.80E-03	4.36E-03	3.76E-02
BECG	12042	6805	33459	1000	1.20E+01	6.81E+00	3.35E+01
COC	4.35	5.48	32.3	1000	4.35E-03	5.48E-03	3.23E-02
KET	0.72	1.13	8.34	1000	7.20E-04	1.13E-03	8.34E-03
MAMP	1.97	2.51	20.51	1000	1.97E-03	2.51E-03	2.05E-02
MDMA	0.86	0.22	24.18	1000	8.60E-04	2.20E-04	2.42E-02
THC-COOH	0.034	0.029	0.115	1000	3.40E-05	2.90E-05	1.15E-04

LC 50: Half maximal letal concentration estimated by ECOSAR; EC50: Half maximal effective concentration estimated by ECOSAR; PNEC: predicted non effect concentration.

PNEC: calculated by dividing the lowest short-term EC50 (half maximal effective concentration) or LC50 (half maximal letal concentration) by an assessment factor (AF) of 1000 to consider the uncertainty in the obtained laboratory toxicity data when it is extrapolated to the field.

Table S5.4. Linearity values of the method performance.

Compound	IS	Linearity Concentration range (ng L^{-1}): 1-1000	
		Linearing equation	R^2
6-ACMOR	6-ACMOR-d_3	y=1.02x+0.11	0.9901
AMP	AMP-d_5	y=1.59x+0.04	0.9962
BECG	BECG-d_3	y=1.09x+0.06	0.9952
COC	COC-d_3	y=1.08x+0.03	0.9985
KET	KET-d_4	y=2.31x-0.19	0.9984
MAMP	MAMP-d_5	y=2.27x+0.03	0.9999
MDMA	MDMA-d_5	y=1.23x+0.02	0.9977
THC-COOH	THC-COOH-d_3	y=7.23x+0.10	0.9981

Table S5.5. Frequency of detection in the influent of WWTPs of illicit drugs and metabolites (%) and levels quantified (mean and range).

	INFLUENT PINEDO I			INFLUENT PINEDO II			INFLUENT QUART-BENÀGER		
	Freq (%)	Concentration (ng L^{-1})		Freq (%)	Concentration (ng L^{-1})		Freq (%)	Concentration (ng L^{-1})	
		Mean	Range		Mean	Range		Mean	Range
2013									
6-ACMOR	n.d.	<LOQ		n.d.	<LOQ		n.d.	<LOQ	
AMP	100	39.8	23.6-61.7	100	21.2	13.1-35.4	100	59.7	11.2-110.0
BECG	100	1356.0	870.9-1951.9	100	1393.1	879.2-2210.3	100	3050.1	1011.0-7752.5
COC	100	748.5	403.2-1871.7	100	1269.5	434.9-3429.7	100	1696.6	375.1-3291.9
KET	100	21.1	6.1-101.4	100	50.2	6.8-131.8	100	20.1	11.1-45.7
MAMP	100	4.7	3.1-7.0	100	4.1	2.9-6.3	100	4.3	2.2-7.6
MDMA	100	39.4	8.3-101.4	100	25.0	5.7-67.4	100	52.0	7.1-159.1
THC-COOH	100	222	140.7-295.6	100	236.9	136.9-396.9	100	484.9	171.4-940.2
2012									
6-ACMOR	n.d.	<LOQ		6.7	16.6	16.6-16.6	n.d.	<LOQ	
AMP	100	68.2	50.3-86.2	100	55.4	44.3-82.5	100	2.5	1.7-3.3
BECG	100	1909.0	1016.4-3525.1	100	181.9	1204.1 -2972.7	100	3747.4	2058.9-7028.7
COC	100	336.3	195.1-659.3	100	222.1	172.2-322.5	100	178.5	110.3-284.1
KET	100	17.8	11.4-22.4	100	2.9	1.3-6.2	100	1.0	<LOQ-1.1
MAMP	100	48.8	31.5-69.1	100	47.1	41.3-58.1	100	1.7	1.2-2.1
MDMA	100	21.1	7.6-48.6	100	20.3	12.1-38.2	100	1.0	<LOQ-1.0
THC-COOH	100	92.7	60.2-198.6	100	37.6	22.1-60.2	100	60.5	19.2-110.3
2011									
6-ACMOR	n.d.	<LOQ		n.d.	<LOQ		n.d.	<LOQ	
AMP	100	73.7	61.4-98.5	100	57.5	50.5-71.6	100	62.2	48.4-74.0
BECG	100	1815.4	1178.1-2970.7	100	1698.8	1220.5-2755.3	100	3560.5	2292.8-6824.8
COC	100	398.1	245.1-652.4	100	254.0	185.8-350.4	100	336.3	190.3-464
KET	n.a.			n.a.			n.a.		
MAMP	100	51.0	46.8-67.3	100	46.7	44.9-48.4	100	47.4	44.5-51.7
MDMA	100	14.2	8.0-36.8	100	13.5	9.3-23.6	100	12.0	7.3-22.9
THC-COOH	100	71.3	20.4-118.8	100	44.8	22.2-74.8	100	77.7	18.8-165

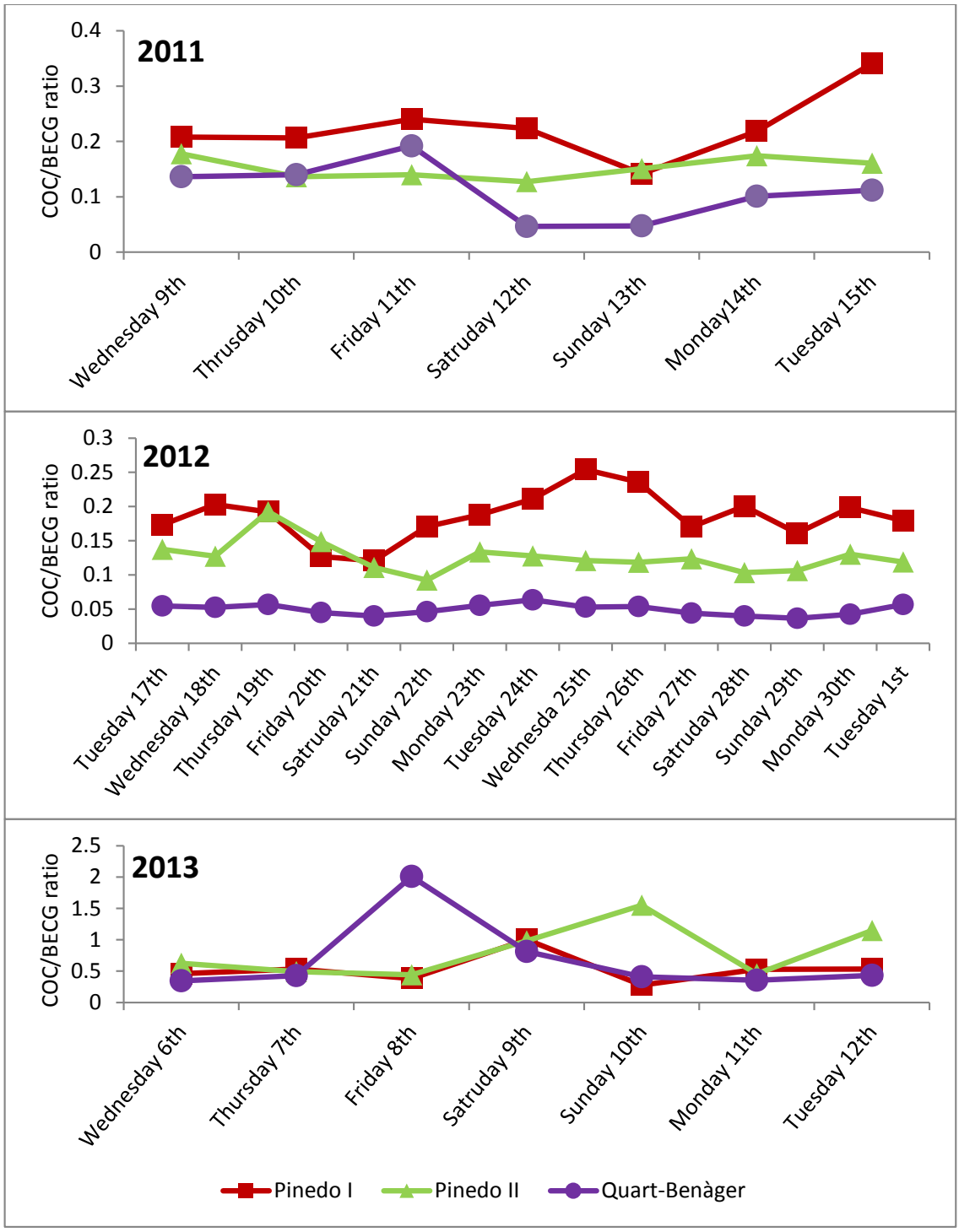

Figure S5.1. COC/BECG ratio based on excretion values of COC and BECG after COC consumption

Table S5.6. Frequency of detection in the effluent of WWTPs of illicit drugs and metabolites (%) and levels quantified (mean and range).

	EFFLUENT PINEDO I			EFFLUENT PINEDO II			EFFLUENT QUART-BENÀGER		
	Freq (%)	Concentration (ng L^{-1})		Freq (%)	Concentration (ng L^{-1})		Freq (%)	Concentration (ng L^{-1})	
		Mean	Range		Mean	Range		Mean	Range
2013									
6-ACMOR	n.d.			n.d.			n.d.		
AMP	n.d.			n.d.			n.d.		
BECG	100	58.3	4.3-115.3	100	17.2	3.1-46.1	100	158	72.8-341
COC	n.d.			n.d.			n.d.		
KET	100	16.9	6.7-24.8	100	13	4.4-20.7	100	19.6	14.3-27
MAMP	n.d.			14.3			n.d.		
MDMA	57.1	50.2	31.1-84.7	57.1	26.7	15.0-43.0	71.4	37.2	8.7-70.1
THC-COOH	n.d.			n.d			n.d.		
2012									
6-ACMOR	n.d.			n.d.			n.d.		
AMP	n.d.			n.d.			n.d.		
BECG	100	79.6	4.2-179.7	100	28.0	5.8-69.9	100	247.0	68.4-755.0
COC	n.d.			n.d.			n.d.		
KET	100	26.5	3.7-43.4	73.3	3.2	1-5.2	93.3	1.0	<LOQ-1.2
MAMP	n.d.			n.d.			n.d.		
MDMA	53.3	18.7	6.5-27.7	60	14.2	4.9-20.2	46.7	1.0	<LOQ-1.2
THC-COOH	n.d.			n.d.			n.d.		

Table S5.7. Risk Quotient calculated from average and maximum concentrations of effluents values of selected WWTP in 2012 and 2013.

Risk Quotients								
Trophic level	WWTP	Year	Average values			Maximum values		
			BECG	KET	MDMA	BECG	KET	MDMA
Algae	Pinedo I	2012	0.000	0.037	0.022	0.000	0.060	0.032
		2013	0.000	0.027	0.043	0.000	0.034	0.098
	Pinedo II	2012	0.000	0.004	0.017	0.000	0.007	0.023
		2013	0.000	0.018	0.031	0.000	0.029	0.050
	Quart-Benàger	2012	0.000	0.001	0.004	0.000	0.002	0.005
		2013	0.000	0.000	0.000	0.000	0.038	0.081
Daphnids	Pinedo I	2012	0.000	0.023	0.085	0.000	0.038	0.126
		2013	0.000	0.015	0.228	0.000	0.022	0.385
	Pinedo II	2012	0.000	0.003	0.065	0.000	0.005	0.092
		2013	0.000	0.012	0.121	0.000	0.018	0.196
	Quart-Benàger	2012	0.000	0.001	0.004	0.000	0.001	0.005
		2013	0.000	0.017	0.169	0.000	0.024	0.318
Fish	Pinedo I	2012	0.000	0.003	0.001	0.000	0.005	0.001
		2013	0.000	0.002	0.002	0.000	0.003	0.004
	Pinedo II	2012	0.000	0.000	0.001	0.000	0.001	0.001
		2013	0.000	0.002	0.001	0.000	0.002	0.002
	Quart-Benàger	2012	0.000	0.000	0.000	0.000	0.000	0.000
		2013	0.000	0.002	0.002	0.000	0.003	0.003

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PUBLICACIÓN CIENTÍFICA 6

Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker

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Estimation of alcohol consumption during "Fallas" festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker

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HIGHLIGHTS

- Direct determination of ethyl sulfate in wastewater by ion-pair LC–MS/MS
- Different ion-pairs and additives were tested and compared.
- Sewage epidemiology was applied to estimate alcohol consumption.
- The increase in the alcohol consumption during Fallas festivity is noticeable.

GRAPHICAL ABSTRACT

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ABSTRACT

Alcohol consumption has been increasing in the last years and it has become a sociological problem due its derived health and safety problems. Ethyl sulfate is a secondary metabolite of the alcohol degradation that is excreted through the urine (0.010–0.016%) after alcohol ingestion and it is quite stable in water. In this study, a new methodology to determine ethyl sulfate by ion-pair liquid chromatography-tandem mass spectrometry (LC–MS/MS) was developed. Different ion-pairs and additives were tested directly in the sample extracts or in the mobile phase. The best ion-pair was set up adding 0.5 M of tributylamine and 0.1% of formic acid to the sample. The limit of quantification was 0.3 μg L^{-1} and the intra-day and inter-day precision of the method were ≤2.8 and ≤3.0%, respectively. Good linearity ($r^2 < 0.999$) and low matrix effect (<30% corrected by using internal isotopically labelled internal standard) were achieved. The sampling campaign was from 4th to 20th March of 2014 covering the festivity of Fallas (15th to 19th March). Ethyl sulfate was determined in all influents of the 3 wastewater treatment plants (Pinedo I, Pinedo II and Quart-Benàger) belonging to Valencia and surrounding area. Ethyl sulfate concentrations ranged from 1.46 to 19.85 μg L^{-1} and alcohol consumption ranged from 1.07 to 56.11 mL day^{-1} $inhab^{-1}$, being the highest value of alcohol consumption determined during Fallas. This study presents a reliable and alternative method to traditional ones to determine alcohol consumption by population that provides real-time information of alcohol consumption.

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1. Introduction

Alcohol is a psychoactive substance widely consumed through the world; in fact, its consumption has been increasing over time. This situation is such that today, alcohol is considered as a serious health and security problem due to the effects associated with their usual consumption. Drinking alcohol produces gastrointestinal and cardiovascular diseases as well as is one of the leading causes of accidents, from domestic to traffic related (OEDT, 2011). Common methods for determining alcohol consumption are based on general population surveys or reports of epidemiological, alcohol expenditure and sales data (WHO, 2014). However, these data are not completely reliable because surveys have important bias of the alcohol consumption. Sales data do not include illicit and informally produced alcohol or do not keep in mind the stockpiling or wastage. Thus, alternative method that helps to improve the estimation of alcohol consumption was needed.

Once the alcohol has been ingested, it is degraded to two minor metabolites, ethyl glucuronide (EtG) and ethyl sulfate (EtS), which are excreted through the urine (0.010–0.016% on molar basis) (Høiseth et al., 2008). Both are determined in clinical and forensic laboratories for the surveillance of abstinence, establishing the alcohol consumption in workplace testing and rehabilitation programmes for alcohol dependence (Thierauf et al., 2010a; Thierauf et al., 2011).

Sewage epidemiology has been successfully applied to estimate the consumption of drugs of abuse based on specific metabolites as a biomarker, which are excreted through the urine in wastewater treatment plants (WWTP) (Andres-Costa et al., 2014; Damien et al., 2014; Ort et al., 2014; Thomas et al., 2012; Vazquez-Roig et al., 2014). EtG is unstable in sewage effluent (Halter et al., 2009). Contrarily, EtS has been demonstrated to be stable in manometric respiratory test — high concentrations of bacteria and EtS — for at least 6 days and up to 28 days in closed bottle test — low EtS and bacteria density from a WWTPs effluent (Halter et al., 2009; Thierauf et al., 2008).

Analytical methods have been described for the determination of EtS in biological matrices such as plasma, serum, vitreous humour, placental and foetal tissues, and meconium (Kummer et al., 2013; Morini et al., 2010; Morini et al., 2007; Thierauf et al., 2010a; Thierauf et al., 2011; Thierauf et al., 2008; Thierauf et al., 2010b) either by gas chromatography–mass spectrometry, liquid chromatography–tandem mass spectrometry (LC–MS/MS), liquid chromatography with pulsed electrochemical detection, capillary zone electrophoresis, or immunochemical test. Otherwise, few bioanalytical methods are available for the determination of EtS in wastewater where a lower detection limit is required (Mastroianni et al., 2014; Reid et al., 2011; Rodríguez-Álvarez et al., 2014). These methods are based on LC–MS/MS exploiting ionic exchange mechanisms because EtS is poorly retained on conventional C_8 and C_{18} reverse phase chromatographic columns (Reid et al., 2011). Methods reported showed that different ion-pairs can be used and can be added to the mobile phase or to the sample. The first method reported used an ion-pair reagent dihexylammonium acetate (DHAAc) into the mobile phase (Reid et al., 2011). More recently, a similar approach based on dibutylammonium acetate (DBAAc) as ion-pair reagent added into the mobile phase was proposed (Mastroianni et al., 2014). Alternatively, method based on tetrabutylammonium bromide (TBAB) ion-pair added to the sample achieves a determination of EtS in wastewater directly (after filtration, internal standard and ion-pair addition) by LC–quadrupole time-of-flight (QqTOF)–MS. This method permits the use of stronger non-volatile amines, such as TBAB, as the amount entering the MS is reduced in comparison to its introduction in the eluent. However, TBAB entering into the MS system also can have an important impact on analyte signal (Rodríguez-Álvarez et al., 2015; Rodríguez-Álvarez et al., 2014).

In this context, the aim of the present study is to develop a simple, fast and reliable method to determine EtS by ion-pair LC–MS/MS. Thus, different ion-pairs have been tested and were added to the sample or mobile phase in order to select the best option. The developed method was applied to calculate the alcohol consumption through the analysis of the influents of 3 WWTPs, Pinedo I, Pinedo II and Quart-Benàger. These WWTPs treat the wastewater of Valencia and its surrounding area. The sampling period was from 4th to 20th March of 2014 including the big festivity of Fallas. That festivity is in honour of Saint Joseph that takes place every year in Valencia from 15th to 19th March. As in many other festivities, heavy drinking is an important part of Fallas fun, and Valencia is plenty of drinking stalls and bars all over the city.

2. Materials and methods

2.1. Chemicals

EtS sodium salt and EtS-d5 sodium salt were obtained from Sigma-Aldrich (Madrid, Spain) as solutions in methanol at a concentration of 1 mg mL^{-1}. Stock standard solutions were prepared at 1 μg mL^{-1} by appropriate dilution of the commercial standards in methanol and were stored in the dark at −20 °C. Working solutions were prepared by diluting stock solution in methanol as on daily basis and stored at 4 °C. EtS-d5 was maintained at a final concentration of 25 μg L^{-1} into the standard calibration solutions and samples.

Tetrabutylammonium chloride (TBAC), diethylamine (DEA), tributylammonium (TBAmm), dihexylamine (DHA) and isopropylamine (IPA) were from Sigma-Aldrich and tributylamine (TBA) from Merk (Schuchardt, Germany). Other reagents and solvents were formic acid (FA) from Amresco-inc (Solon, Ohio, USA) and ammonium formate (AmF) and acetic acid (AcA) from Alfa Aesar GmbH & Co KG (Karlsruhe, Germany). DHAAc was prepared from equimolar volumes of DHA and AcA. Methanol was purchased from Panreac (Madrid, Spain) and ultrapure water obtained from a Milli-Q water purification system.

2.2. Sample collection and treatment

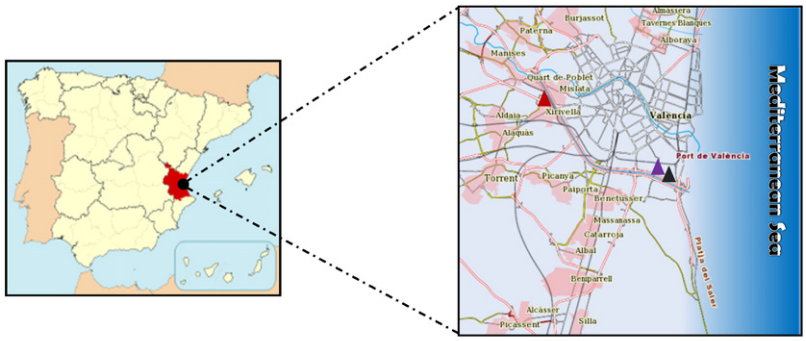
Wastewater samples were collected from influents and effluents of Pinedo I, Pinedo II and Quart-Benàger, that treat about 1,500,000 people and a flow of 355,233 m^3 day^{-1} (EPSAR, 2014). Fig. 6.1 shows the techni-cal characteristics and the location of each WWTP (more information of each WWTP have been presented in Tables S6.1, S6.2 and S6.3 in Supplemen-tary information). The sampling was conducted from 4th to 20th March of 2014.

Wastewater samples were provided by WWTP operators and were arranged in 1 L polypropylene bottles, previously rinsed with ultrapure water and wastewater samples prior to the wastewater collection. The samples were transported back to the laboratory in a dark and iced cool box. Once at the laboratory, aliquots of 15 mL of wastewater samples were frozen at −20 °C until analysis. Then the samples were thawed and prepared in 2 mL amber vials appropriately. The best ion-pair was set up adding 0.5 M of TBA and 0.1% of FA to the sample.

2.3. Liquid chromatography–mass spectrometry (LC–MS/MS)

Chromatographic separation of EtS was performed using an Agilent Technologies 1260 Infinity Ultra High-Performance Liquid Chromatograph (UHPLC). The column was Kinetex C18 (1.7 μm, 100 Å, 50 × 2.10 mm) and it was maintained at temperature of 30 °C and a constant flow rate of 0.2 mL min^{-1}. The isocratic mobile phase was 90% eluent A (FA 0.1% in Milli-Q water) and 10% eluent B (FA 0.1% in methanol). The injected volume of sample was 5 μL. Methanol–water (10: 90 v/v) both with (1) 7 mM DHAAc; (2) 10 mM DEA, 10 mM AmF and 10 mM AcA; and (3) 10 mM TBA, 10 mM AmF and 10 mM AcA.

The UHPLC was coupled to an Agilent Technologies 6410 triple quadrupole mass spectrometer with an electrospray Turbo V ionization source working in negative ionization (ESI^-) mode, 300 °C gas temperature, 11 L min^{-1} gas flow and 25 psi nebulizer. Quantifier and qualifier



	Total installed power (kW)	Project flow (m^3 day^{-1})	Treat flow (m^3/day^{-1})	Population served (Inhab)	Efficiency (%) SS	BOD	COD
▲ Pinedo I	3,378	124,800	101,674	335,825	93	94	91
▲ Pinedo II	15,232	200,000	219,774	982,264	98	98	96
▲ Quart-Benàger	2,300	60,000	33,785	154,421	98	98	95

Inhab: inhabitants; SS: suspended solids; BOD: biology oxygen demand; COD: chemical oxygen demand

Fig. 6.1. Location and characteristics of each WWTP.

transitions were optimized by selected reaction monitoring (SRM). Specific parameters as collision energy (CE) and fragmentor (F) were optimized for EtS and EtS-d5 separately. EtS presents a precursor ion $[M–H]^-$ m/z of 125 and a product ion corresponding m/z 97 $[HSO_4]^-$ (quantifier transition) with a CE of 13 and F of 71 and m/z 80 $[SO_3]^-$ (qualifier transition) with a CE of 37 and F of 71. EtS-d5 presents a precursor ion $[M–H]^-$ m/z 130 and a corresponding product ions at m/z 98 $[DSO_4]^-$ (quantifier transition) with a CE of 5 and F of 71 and m/z 80 $[SO_3]^-$ (qualifier transition) with a CE of 33 and F of 71. Retention time of EtS and EtS-d5 was 3.805 min.

2.4. Validation of the analytical method

Parameters as linearity, limit of quantification, limit of detection, precision as repeatability and reproducibility, and matrix effect were studied to validate the analytical method. Linearity was evaluated by preparing 9-points calibration curve of EtS within the range of 0.1–500 μg L^{-1}. Each point was injected in triplicate. Calibration curve was established using linear regression and was qualified by the linear correlation coefficient r^2.

Limit of detection (LOD) and limit of quantification (LOQ) of the method were determined experimentally by injection of decreasing concentrations of the standard mixture, as the amount of analyte that gave a signal-to-noise ratio of 3:1 and 10:1, respectively.

The precision of the method was evaluated by repeatability and reproducibility studies and they were expressed as the relative standard deviation (RSD). Intra and inter-day precision of the analytical method was carried out covering 3 concentrations (1, 50 and 100 μg L^{-1}) of the standard 5 times on the same day or on 5 different days, respectively.

Matrix effect (ME) was evaluated by spiking ultrapure water and wastewater samples with concentrations ranging from 0.1–500 μg L^{-1} of EtS. ME was determined using the following equation (Eq. (6.1)):

$$\mathrm{ME}\,(\%) = \frac{\text{Calibration Graph Slope standard in wastewater}}{\text{Calibration Graph Slope standard in water}} \times 100 \qquad (6.1)$$

If ME (%) < 100 means that there is a signal suppression, and therefore there are losses in the concentration detected in the matrix. Otherwise, if ME (%) > 100, there is an amplification of the signal.

In addition, solvent blanks containing methanol were prepared to run after every ten samples to check any potential contamination occurring during the extraction of samples.

2.5. Alcohol consumption estimation

Alcohol consumption was back-calculated from daily measured loads of drugs target residues, using the model suggested by Zuccato et al. (2005) with the following equation (Eq. (6.2)):

$$Q\left(\mathrm{mL\ day^{-1}\ inh^{-1}}\right) = C * FC * FR * \frac{1}{\mathrm{Ninh}} * \frac{100 * MR}{Mex} * \frac{1}{\rho} \qquad (6.2)$$

where Q is the load of alcohol consumption; C is the concentration of each sample; FC is the correction factor for stability; FR is the flow rate; Ninh is the number of inhabitants (>15 years old) (INE, 2014) linked to the WWTPs, population size was normalized using hydrochemical parameters as chemical oxygen demand (COD); MR is the molar ration between ethanol and EtS; Mex is the percentage of metabolic excretion; and ρ is alcohol density.

3. Results

3.1. LC–MS/MS

The EtS is a polar compound being poor retained in conventional reversed phase chromatography as reported in the literature (Reid et al., 2011). Alternative polar HILIC columns have provided neither better retention nor improved peak shape (Rodríguez-Álvarez et al., 2014). There are only two studies that determined EtS in wastewater through LC–MS/MS employing ion-pairs as DHAAc (7 mM) and DBAAc (5 mM) added to the mobile phase (Mastroianni et al., 2014; Reid et al., 2011). Fig. 6.2a examines the effects of varying ion-pair reagent on the retention of EtS using 7 mM of DHAAc, 10 mM of DEA with 10 mM of AmF and AcA and 10 mM of TBA with 10 mM of AmF and AcA in the mobile phase

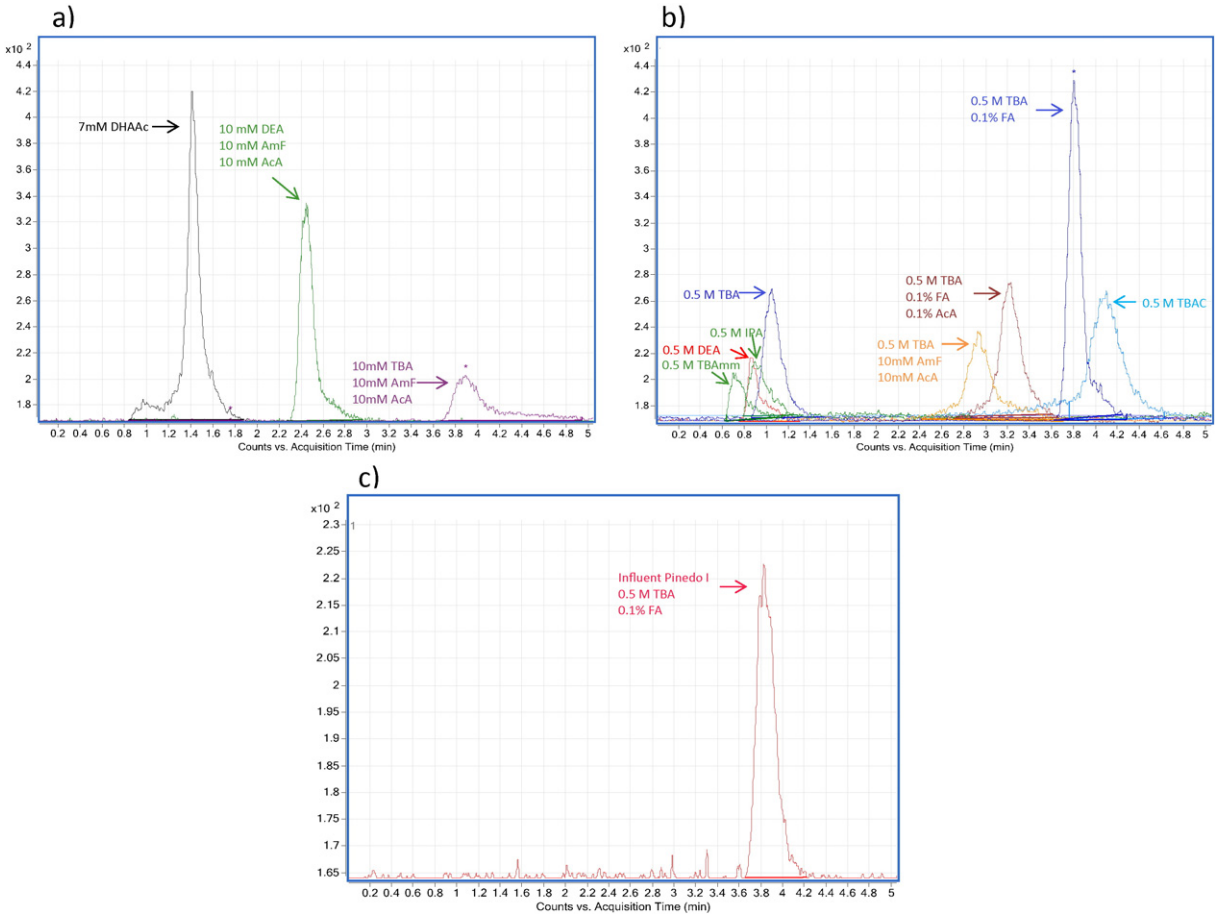


Fig. 6.2. LC–MS/MS chromatogram of EtS (100 μg L^{-1}) with different ion-pairs: a) 7 mM DHAAc, 10 mM DEA + 10 mM AmF + 10 mM AcA and 10 mM TBA + 10 mM AmF + 10 mM AcA in the mobile phase; b) 0.5 M of TBAmm, DEA, IPA, TBA, TBAC, TBA + 0.1% FA; TBA + 0.1% FA + 0.1% AcA and TBA + 10 mM AmF + 10 mM AcA in the sample; c) influent sample of Pinedo I with 0.5 M TBA + 0.1% FA.

(methanol and water). A disadvantage of the use of ammonium as a mobile phase buffer is that it reduces the ESI–MS ionization efficiency, thereby limiting the sensitivity of the analysis (Rodríguez-Álvarez et al., 2014). The Kinetex C_{18} column with core-shell technology retains some of the ionic pairs. Proper peak shape but low retention was obtained for DHAAc while higher retention and poor peak shape were obtained with DEA and TBA. Another disadvantage of adding the ion-pair into the mobile phase is that the mass spectrometer gets dirty easily.

In the electrostatic model of retention through ion-pairing, the hydrophobic ion-pair reagents are first adsorbed onto the surface of the hydrophobic stationary phase where, in the case of this study, the EtS interact electrostatically with the positive charge. Then, the ion-pair can also be added to the sample as established. Fig. 6.2b shows chromato-grams of different ion-pairs, added to the sample, tested in this study as TBAmm, DEA, IPA, TBA, and TBAC. Different amounts of ion-pair were tested 50, 100, 200, 500 and 1000 mM and the best results were obtain-ed making the extract 500 mM with the ion-pair. The best ion-pair se-lected was TBA due its higher sensitivity and better peak shape. Also, some additives as FA, AcA and AmF were added. The better peak shape and retention time presented was the ion-pair formed by 0.5 M TBA + 0.1% FA added to the sample. Poor chromatograms with early peaks (low retention time) that are broad (large width) were obtained for TBAmm, DEA, IPA and TBA while excellent chromatograms with late-appearing peaks (large retention time) that are still very narrow (small width) were observed with TBA just adding 0.1% of FA. The addition of the ion-pair to the sample keeps the mass spectrometer cleaner that means a clear advantage of this method. Furthermore, TBAmm even that was used in other studies is a non-volatile ion-pairing reagent and when applied to large sample batches, it gave technical problems with EtS ionization because the ESI capillary gets dirty.

3.2. Validation of the analytical method

Calibration curve (9-points) was constructed and linear responses were obtained ($r^2 \geq 0.999$) for EtS in the applied concentration range of 0.1–500 μg L^{-1} using EtS-d5 as internal standard.

Table 6.1 Concentration of EtS in the wastewater samples.

	Influent Pinedo I			Influent Pinedo II			Influent Quart-Benàger		
	Freq	Concentration		Freq	Concentration		Freq	Concentration	
		Mean	Range		Mean	Range		Mean	Range
	(%)	μg L^{-1}	μg L^{-1}	(%)	μg L^{-1}	μg L^{-1}	(%)	μg L^{-1}	μg L^{-1}
EtS	100	7.04	1.46–19.85	100	4.87	1.58–9.89	100	6.40	2.00–10.71

Freq: frequency.

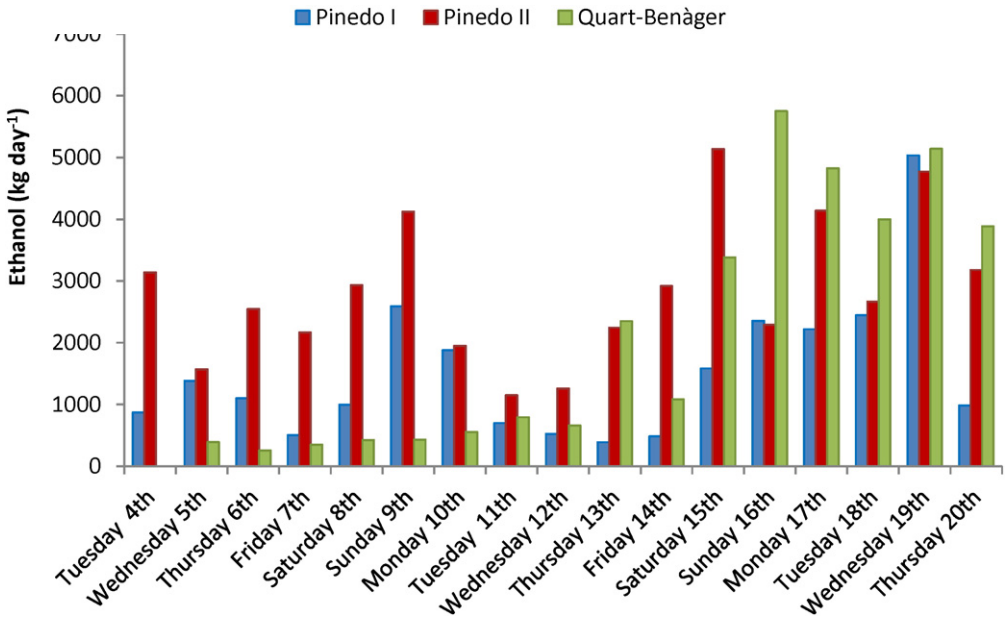


Fig. 6.3. Loads (kg day^{-1}) of pure ethanol during sampling campaign in the 3 WWTPs.

LOD and LOQ of the method for EtS that gave a signal-to-noise ratio (S/N) > 3 and 10, respectively, were 0.1 μg L^{-1} for LOD and 0.3 μg L^{-1} for LOQ. Results of precision expressed as RSD were 2.81% for intra-day precision and 2.97% for inter-day precision. Both results were in concordance with the data reported by Reid et al. (2011), Rodríguez-Álvarez et al. (2014) and Mastroianni et al. (2014).

There is a signal suppression of EtS in wastewater matrix, being the ME equal to 73.02%. This signal suppression was solved with the addition of EtS-d5 to wastewater samples.

3.3. Concentration of EtS in wastewater and alcohol consumption by population

The developed method was applied to the determination of EtS in samples collected from WWTPs of Pinedo I, Pinedo II and Quart-Benàger belonging to Valencia and surrounding area from 4th to 20th March 2014 period (Fallas festivity took place from 15th to 19th March of 2014). The period between Tuesday 4th to Friday 14th is considered as a normal week and the period from Saturday 15th to Thursday 20th is considered as an unusual week due the celebration of Fallas.

Table 6.2
Estimation of collective alcohol use during the period from 4th to 20th March of 2014.

	Alcohol consumption (mL day^{-1} inhabitants^{-1}; >15 years old)		
	WWTP Pinedo I	WWTP Pinedo II	WWTP Quart-Benàger
Tuesday 4th	4.83	4.47	n.a.
Wednesday 5th	7.70	2.14	3.31
Thursday 6th	6.48	3.18	3.64
Friday 7th	3.08	2.80	4.94
Saturday 8th	5.96	4.89	6.01
Sunday 9th	18.31	6.44	6.10
Monday 10th	11.55	2.47	4.43
Tuesday 11th	3.84	1.52	5.74
Wednesday 12th	1.11	1.07	3.69
Thursday 13th	2.44	1.86	12.38
Friday 14th	2.78	5.25	8.66
Saturday 15th	13.83	6.89	26.99
Sunday 16th	16.53	4.35	45.87
Monday 17th	13.90	6.89	52.68
Tuesday 18th	13.36	4.71	31.90
Wednesday 19th	23.81	9.07	56.11
Thursday 20th	4.86	4.40	31.58

n.a.: not analysed.

Table 6.1 summarizes frequency, concentration range and average values of EtS in the 3 WWTPs. EtS was present in 100% of influents in the 3 WWTPs and ranged from 1.46 μg L^{-1} to 19.85 μg L^{-1}. The highest average concentration detected was in Pinedo I (7.04 μg L^{-1}), followed by Quart-Benàger (6.40 μg L^{-1}) and Pinedo II (4.87 μg L^{-1}). Detailed data of EtS concentration detected in each WWTP can be observed in Tables S6.4, S6.5, S6.6 and Fig. S6.1 in Supplementary information. These results are in agreement with data reported in Santiago de Compostela, Oslo and Barcelona, where the EtS concentrations were between 4 and 12, 2–30 and 5–32 μg L^{-1}, respectively (Mastroianni et al., 2014; Reid et al., 2011; Rodríguez-Álvarez et al., 2014).

In the same way as influent samples, the effluent samples of Pinedo I, Pinedo II and Quart-Benàger were processed. The concentration of EtS was <LOD in all effluent samples. So, it is considered that this metabolite has been degraded during the WWTP process. This fact was demon-strated by the study carried out by Reid et al. (2011) where the stability of EtS in wastewaters was of 18 h until half degradation of the concentration.

Daily wastewater flow provided by each WWTP combined with ex-cretion rate of EtS (0.01–0.016%) (Høiseth et al., 2008) were used to cal-culate the load of pure ethanol in kg day^{-1} (Tables S6.4, S6.5 and S6.6 in Supplementary information). Large variation in the load of pure ethanol was observed over the sampling campaign period. These results ranged from 390 to 5033 kg day^{-1} in Pinedo I, 1150 to 5138 g day^{-1} in Pinedo II and 258 to 5749 kg day^{-1} in Quart-Benàger. As it can be observed in Fig. 6.3, there is an increase of amount of pure ethanol that takes place during the weekend (Friday 7th–Monday 10th) in Pinedo II, and in a lesser extent in Pinedo I. Quart-Benàger did not show this trend. This last WWTP is the most complex because there are a number of industri-al effluent and the population declines at the weekend. For the period from Saturday 15th to Thursday 20th an unusual increase can be ob-served in weekdays, being the values of these days greater than values of the same day of the normal week.

Alcohol consumption was calculated using Eq. (6.2) described in Section 2.5. Detailed alcohol consumptions are summarized in Table 6.2. The obtained data of this study ranged from 1.11 to

23.81 mL day^{-1} inh^{-1} in Pinedo I, from 1.07 to 9.07 mL day^{-1} inh^{-1} in Pinedo II and from 3.31 to 56.11 mL day^{-1} inh^{-1} in Quart-Benàger. These results are in good agreement with data obtained in Santiago de Compostela, Barcelona and Oslo (Mastroianni et al., 2014; Reid et al., 2011; Rodríguez-Álvarez et al., 2014). Once again a higher increment in alcohol consumption during weekends in the normal week can be seen. Alcohol consumption increases from 6.48 mL day^{-1} inh^{-1} on Thursday 6th to 18.31 mL day^{-1} inh^{-1} on Sunday 9th in Pinedo I while Pinedo II presents an increase from 3.18 mL day^{-1} inh^{-1} to 6.44 mL day^{-1} inh^{-1} on the same days. Regarding Quart-Benàger the increase occurs from 3.64 mL day^{-1} inh^{-1} on Thursday 4th to 6.10 mL day^{-1} inh^{-1} on Sunday 9th, although an odd value exists in the Quart-Benàger on Thursday 13th when the alcohol consumption reaches a value of 12.38 mL day^{-1} inh^{-1}, being a possible cause some exceptional event in this area regarding to Fallas festivity. There is an exceptional behaviour and clear evidence that alcohol consumption increases just at the beginning of Fallas festivity and it was increasing along the week. Differences between weekend (20.88 mL day^{-1} inh^{-1}) and weekdays (19.98 mL day^{-1} inh^{-1}) were not observed in Fallas week. Maximum values were reaching on the last day Wednesday 19th March, so called “Nit de la Cremà”, with values were 23.81, 9.07 and 56.11 mL day^{-1} inh^{-1} in Pinedo I, Pinedo II and Quart-Benàger, respectively, being these values reached double the levels recorded in this study. See Fig. S6.2 in Supplementary information to have a bet-ter appreciation. The recorded alcohol per capita consumption (>15 years old) by type of alcoholic beverage according to data from 2010 is distributed in 50% of beer, 28% of spirits, 20% of wine and 2% of other (WHO, 2014). Keeping in mind that beer, spirits and wine have 5, 40 and 12% of pure alcohol, respectively, and the most common volumes of these beverages are 250 mL of beer, 30 mL of spirit and 125 mL of wine, each one have 12.5, 12 and 15 mL of pure ethanol, respectively. In this way we can estimate the number of these beverages that people consume. During a normal week, alcohol consumption, at weekdays is approximately less than a half of one portion of each beverage, 85, 10.5 and 35 mL day^{-1} inh^{-1} of beer, spirit and wine, respectively, while during weekend is a little more than half ration of beer, spirit or wine (147, 18.4 and 61.3 mL day^{-1} inh^{-1}, respectively). The alcohol consumption increases in Fallas week markedly, reaching almost two portions of beer (400 ml day^{-1} inh^{-1}) or one portion and a half of spirit or wine (50 and 166.7 mL day^{-1} inh^{-1}, respectively). According to the Global status report on alcohol and health 2014 of the WHO (2014), the estimated average alcohol consumption per inhabitant (>15 years old) in Spain is 16.4 L of pure ethanol per year, approximately 45 mL day^{-1} inh^{-1}. Small deviation can be originated because this study is restricted to Valencia whereas the World Health Organization report was for all Spanish territory.

4. Conclusion

A methodology based on sewage epidemiology by ion-pair LC–MS/MS in negative mode has been developed. It has been applied to influent and effluent samples collected from 3 WWTPs (Pinedo I, Pinedo II and Quart-Benàger) that treat wastewater from Valencia and surrounding area. EtS was detected in all influent samples at concentrations ranging from 1.46 to 19.85 μg L^{-1}. EtS has not been detected in any effluent samples. Alcohol consumption ranged from 1.11 to 23.81 mL day^{-1} inh^{-1} in Pinedo I, from 1.07 to 9.07 mL day^{-1} inh^{-1} in Pinedo II and from 3.31 to 56.11 mL day^{-1} inh^{-1} in Quart-Benàger, it was increasing at weekends in a normal week as it was expected. There is unusual alcohol consumption during Fallas festivity where the alcohol consumption increased during this period reaching its maximum value on Wednesday 19th March, being 23.81, 9.07 and 56.11 mL day^{-1} inh^{-1}in Pinedo I, Pinedo II and Quart-Benàger.

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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.2015.09.126.

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Supplementary information

Estimation of alcohol consumption during “Fallas”festivity in the wastewater of Valencia city (Spain) using ethyl sulfate as a biomarker

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Table S6.1. Data sheet of WWTP Pinedo I.

Pinedo I		
Treatment	**Water**	Pretreatment: grating thick, grating fine, desander and degreaser Primary treatment: physical-chemical and decantation Secondary treatment: activated sludge Tertiary treatment: coagulation floculation and filtration Disinfection treatment: UV
	Sludge	Thickener: gravity, flotation Stabilization: anaerobic Dehydration: centrifuge Sludge post-treatment: / Power generation: cogeneration

SS: suspended solids; BOD: biology oxygen demand; COD: chemical oxygen demand

Table S6.2. Data sheet of WWTP Pinedo II.

Pinedo II		
Treatment	**Water**	Pretreatment: grating thick, grading, desander and degreaser Primary treatment: physical-chemical and decantation Secondary treatment: activated sludge Tertiary treatment: coagulation floculation and filtration Disinfection treatment: UV
	Sludge	Thickener: gravity, flotation Stabilization: anaerobic Dehydration: centrifuge Sludge post-treatment: incineration Power generation: cogeneration

SS: suspended solids; BOD: biology oxygen demand; COD: chemical oxygen demand

Table S6.3. Data sheet of WWTP Quart-Benàger.

Quart-Benàger		
Treatment	**Water**	Pretreatment: grating thick, grating fine, grading, homogenezation tank desander and degreaser Primary treatment: physical-chemical and decantation Secondary treatment: activated sludge and phosphorus removal Tertiary treatment: coagulation floculation and filtration Disinfection treatment: UV
	Sludge	Thickener: gravity, flotation Stabilization: anaerobic Dehydration: centrifuge Sludge post-treatment: drying heat

SS: suspended solids; BOD: biology oxygen demand; COD: chemical oxygen demand

Table S6.4. Concentration of EtS in wastewater samples, ethanol loads and alcohol consumption in WWTP of Pinedo I.

WWTP Pinedo I			
	EtS ($\mu g\ L^{-1}$)	Ethanol load ($kg\ day^{-1}$)	Alcohol consumption ($mL\ day^{-1}\ inh^{-1}$)
Tuesday 4th	3.50	872	4.83
Wednesday 5th	6.46	1383	7.70
Thursday 6th	5.19	1100	6.48
Friday 7th	2.88	506	3.08
Saturday 8th	5.42	997	5.96
Sunday 9th	14.90	2591	18.31
Monday 10th	8.44	1881	11.55
Tuesday 11th	2.83	700	3.84
Wednesday 12th	2.06	525	1.11
Thursday 13th	1.46	390	2.44
Friday 14th	2.75	482	2.78
Saturday 15th	10.11	1585	13.83
Sunday 16th	10.97	2356	16.53
Monday 17th	9.55	2220	13.90
Tuesday 18th	9.15	2444	13.36
Wednesday 19th	19.85	5033	23.81
Thursday 20th	4.08	982	4.86

Table S6.5. Concentration of EtS in wastewater samples, ethanol loads and alcohol consumption in WWTP of Pinedo II.

WWTP Pinedo II			
	EtS ($\mu g\ L^{-1}$)	Ethanol load ($kg\ day^{-1}$)	Alcohol consumption ($mL\ day^{-1}\ inh^{-1}$)
Tuesday 4th	5.85	3138	4.47
Wednesday 5th	2.74	1570	2.14
Thursday 6th	4.43	2546	3.18
Friday 7th	3.58	2166	2.80
Saturday 8th	5.26	2932	4.89
Sunday 9th	8.00	4123	6.44
Monday 10th	3.17	1950	2.47
Tuesday 11th	1.58	1150	1.52
Wednesday 12th	1.80	1259	1.07
Thursday 13th	2.53	2245	1.86
Friday 14th	3.83	2917	5.25
Saturday 15th	7.63	5138	6.89
Sunday 16th	4.00	2290	4.35
Monday 17th	7.23	4140	6.89
Tuesday 18th	5.31	2666	4.71
Wednesday 19th	9.89	4772	9.07
Thursday 20th	5.92	3179	4.40

Table 6.6. Concentration of EtS in wastewater samples, ethanol loads and alcohol consumption in WWTP of Quart-Benàger.

	WWTP Quart-Benàger		
	EtS ($\mu g\ L^{-1}$)	Ethanol load ($kg\ day^{-1}$)	Alcohol consumption ($mL\ day^{-1}\ inh^{-1}$)
Tuesday 4th	n.a	n.a	n.a
Wednesday 5th	5.87	391	3.31
Thursday 6th	3.80	258	3.64
Friday 7th	5.04	350	4.94
Saturday 8th	7.17	426	6.01
Sunday 9th	7.00	432	6.10
Monday 10th	6.73	556	4.43
Tuesday 11th	3.79	793	5.74
Wednesday 12th	2.00	661	3.69
Thursday 13th	4.71	2351	12.38
Friday 14th	5.33	1085	8.66
Saturday 15th	9.79	3383	26.99
Sunday 16th	10.71	5749	45.87
Monday 17th	8.23	4826	52.68
Tuesday 18th	7.16	3997	31.90
Wednesday 19th	9.58	5140	56.11
Thursday 20th	5.34	3887	31.58

n.a. not analysed.

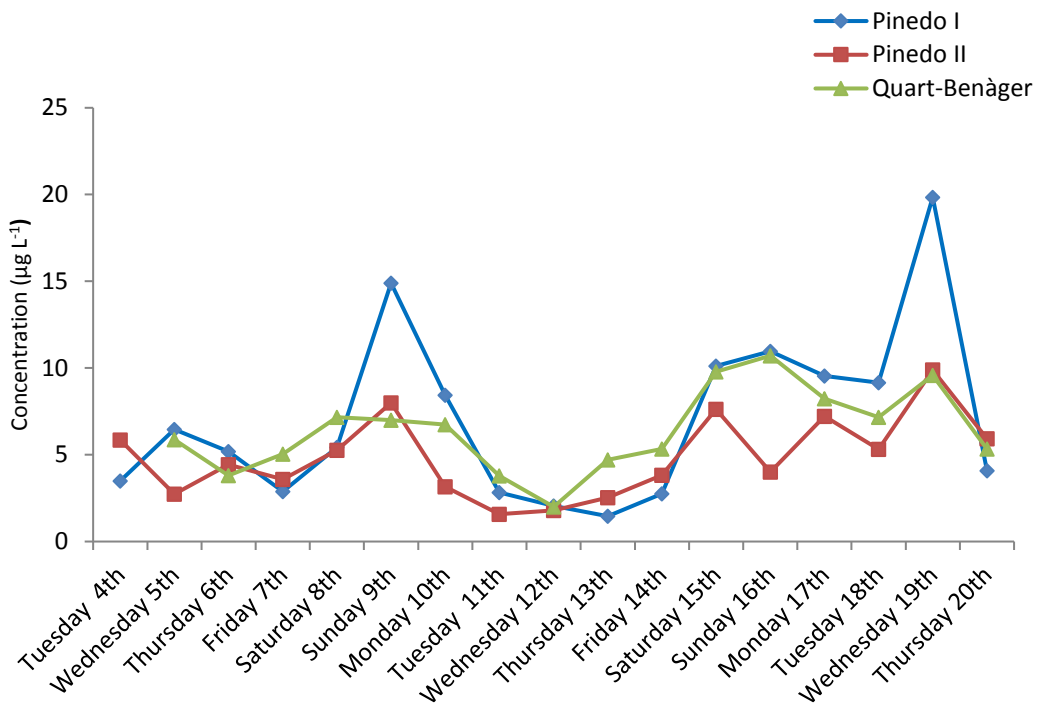

Fig. S6.1. Daily average concentrations of EtS (µg L^{-1}) of Pinedo I, Pinedo II and Quart-Benàger during sampling campaign.

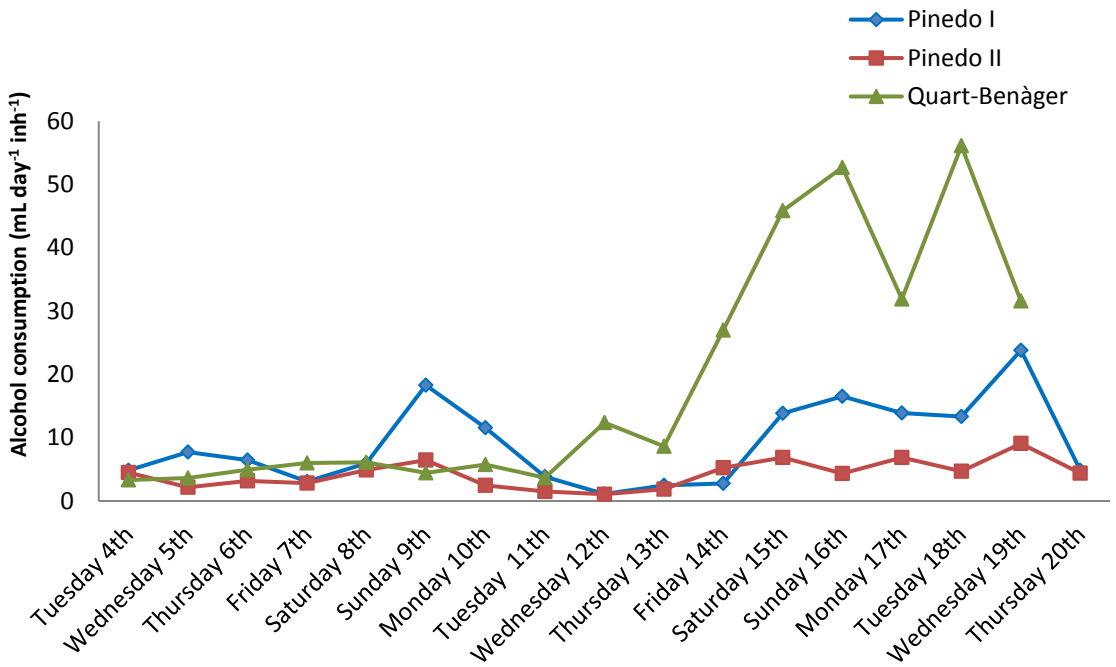

Fig. S6.2. Estimation of collective alcohol consumption for WWTP of Pinedo I, Pinedo II and Quart-Benàger during sampling campaign.

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Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study

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Estimating population size in wastewater-based epidemiology. Valencia metropolitan area as a case study

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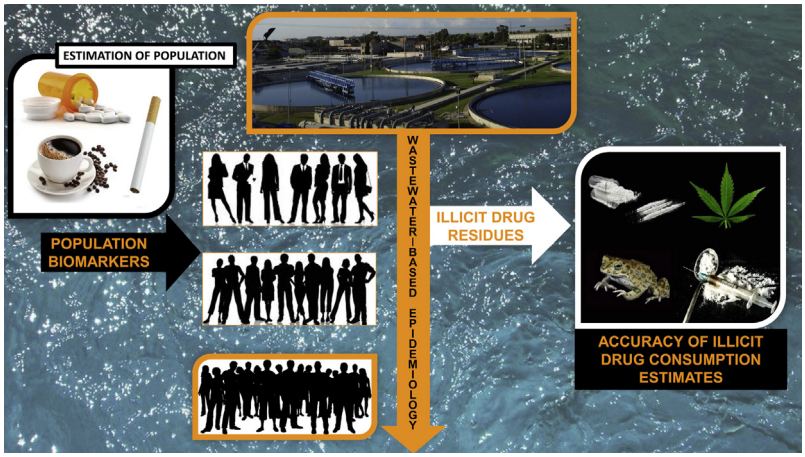
HIGHLIGHTS

- Dilute and shoot or SPE followed by LC–MS/MS to analyze 10 human biomarkers.
- Population served by WWTP estimated from these biomarkers.
- Comparison to population estimates based on WWTP capacity, COD, BOD, N and P.
- Consumption of several drugs calculated using these biomarkers.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater can provide a wealth of epidemiologic data on common drugs consumed and on health and nutritional problems based on the biomarkers excreted into community sewage systems. One of the biggest uncertainties of these studies is the estimation of the number of inhabitants served by the treatment plants. Twelve human urine biomarkers —5-hydroxyindoleacetic acid (5-HIAA), acesulfame, atenolol, caffeine, carbamazepine, codeine, cotinine, creatinine, hydrochlorothiazide (HCTZ), naproxen, salicylic acid (SA) and hydroxycotinine (OH—COT)— were determined by liquid chromatography-tandem mass spectrometry (LC–MS/MS) to estimate population size. The results reveal that populations calculated from cotinine, 5-HIAA and caffeine are commonly in agreement with those calculated by the hydrochemical parameters. Creatinine is too unstable to be applicable. HCTZ, naproxen, codeine, OH—COT and carbamazepine, under or overestimate the population compared to the hydrochemical population estimates but showed constant results through the weekdays. The consumption of cannabis, cocaine, heroin and bufotenine in Valencia was estimated for a week using different population calculations.

1. Introduction

Wastewater-based epidemiology approach (WBE) —based on human health biomarkers excreted by urine and feces that end

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up in sewage– is being increasingly investigated as a prominent tool to estimate the population habits (human health, nutritional status and substance consumption). Up to now, its most well-known application is the estimation of illicit drugs consumption, proposed by Daughton in 2001 [1] and implemented for the first time in Italy by Zuccato et al. [2] (See Fig. S7.1 in the Supplementary content for a schematic overview). Since then, it has been applied successfully to obtain direct estimates of drug abuse all over the world [3,4,5]. However, in a near future, WBE is also envisaged as a promising tool for the real-time collection of exposure/effects data that reflects the overall average health of entire communities [6]. Some of these potential applications include the measurement of isoprostanes to quantify systemic oxidative stress [7,8] or the detection of infectious diseases and pathogens [9]. One of the uncertainties of this approach is the number of people in a certain catchment because a change in population size or the use of not very updated data may radically increase or decrease the estimation of per capita daily consumption. The number of inhabitants (inh) served by the wastewater treatment plant (WWTP) has been commonly estimated from the design capacity of the WWTP, census population data or hydrochemical parameters [10]. Design capacity is, frequently, an untrustworthy parameter, because the WWTP can work below or over this capacity. Census data are based on a fixed population size, according to home address, which usually are not updated and do not account for changes due to tourism or commuting patterns. Population estimates are also calculated using hydrochemical parameters, such as chemical oxygen demand (COD), biological oxygen demand (BOD), total Nitrogen (N) or total Phosphorous (P) [11]. However, these parameters not only reflect the residue from human metabolism but also any biodegradable substance that enters into the sewer system, being altered by industrial discharges, agricultural activities, food waste, etc [12]. Ammonium has also been measured as an indirect marker of urine yielding lower population values than those derived from COD, P and census data, but still unable to distinguish human from non-human contributions [13].

Recently, human biomarkers, such as creatinine, coprostanol, cholesterol, pharmaceuticals or food additives, have been proposed to detect day-to-day fluctuations in the population size [12]. Chiaia et al. [14] first implemented creatinine as a urine biomarker to estimate population size. This substance together with cotinine and caffeine were at high concentrations in WWTPs. Lai et al. [15] did not observe correlation between illicit drugs and pharmaceuticals or acesulfame loads but identified atenolol as an appropriate candidate. Later on, the same group calculated chemical loads of caffeine and several pharmaceuticals, obtaining an estimation higher than the census population but a similar drug consumption pattern [16]. Brewer et al. [17] found that normalization with creatinine as population biomarker changed the between-day trends. Recently, Chen et al. [18], analyzed creatinine, cholesterol, coprostanol, cotinine, cortisol, androstenedione and 5-hydroxyindoleacetic acid (5-HIAA) as population biomarkers. Cholesterol and coprostanol showed a strong affinity to particulate matter, and creatinine, cortisol and androstenedione were also disqualified for stability reasons. Cotinine and 5-HIAA correlated well with the census population. O'Brien et al. [19], measured acesulfame, caffeine and several pharmaceutical in 10 WWTPs using samples collected on census day, obtaining strong correlations between census population and mass loads. Senta et al. [20] found good agreement between population estimated with nicotine metabolites and census population. For these estimations, some additional information, such as annual consumption of these products and excretion rates, is required to determine the quality of the estimations.

This study examined 12 human biomarkers directly determinable by liquid chromatography-tandem mass spectrometry (LC–MS/MS) for the quantitative assessment of population size in wastewater catchments serving the metropolitan area of Valencia city (Spain). The partial objectives of this study were: (1) to develop analytical methods for the 12 population biomarkers; (2) to quantify them in wastewater; (3) to establish which population biomarkers are able to provide solid data on population size comparing results to those obtained from WWTP design capacity, N, P, BOD and COD, and (4) to estimate four illicit drugs consumption using the population size calculated in different ways. To our knowledge, this is the first attempt to establish those that provide the most reliable back-calculations in WBE using easily available data.

2. Experimental

2.1. Chemicals and reagents

High Performance Liquid Chromatography (HPLC) grade methanol (MeOH) was obtained from Prolabo (Barcelona, Spain) and formic acid from Amresco (Solon, OH, USA). Ultrapure water was produced by an Elix Milli-Q Unit (Millipore, USA).

The compounds tested as population biomarkers were selected considering the available information on their human metabolism; exogenous sources; variability of the population excretion rate; stability and presence in sewage; results obtained in previous studies reported in the introduction; and possibility to determine them in the same analytical run as the illicit drugs, by LC–MS/MS in order to optimize resources (description of the general properties of all analytes studied is provided in text and Table S7.1 of the Supplementary content). Analytical standards for hydrochlorothiazide (HCTZ), 5-HIAA, cotinine, caffeine, creatinine, naproxen, salicylic acid (SA) and atenolol were purchased from Sigma-Aldrich (MO, USA). Carbamazepine (CBZ) was purchased from Fluka (Steinheim, Germany). Codeine, bufotenine, morphine, cocaethylene (CET), cocaethylene-d3 (CET-d3) and morphine-d3 were purchased from LGC Standards (Middlesex, UK). Acesulfame K was from Supelco (Pennsylvania, USA). Hydroxycotinine (OH—COT), 11-nor-9-carboxy-tetrahydrocannabinol (THC—COOH) and THC—COOH-d3 were from Cerilliant (TX, USA). All compounds were in solid form with high purity (>99%), except illicit drugs, their deuterated analogs, and OH—COT, which were in single component solutions (100 μg/mL in MeOH). Standard compounds were stored according to supplier's recommendation.

Stock solutions of individual compounds and a mixture solution at a concentration level of 10 μg/L were prepared in MeOH. Calibration solutions were diluted with MeOH—H_2O (10:90, v/v) from the mixture solution. All solutions were stored at −20 °C.

2.2. Sample collection

Samples were collected from three WWTP –Pinedo I, Pinedo II and Quart-Benàger– that cover the whole metropolitan area of Valencia (Spain). These treat up to 400,000 m^3/day of municipal wastewater serving 1.5 million people (information about the WWTPs characteristics is in Table S7.2 of the Supplementary content). Pinedo I and II only receive urban waters and Quart-Benàger receives 40% of urban and 60% of industrial waters.

An automatic time-proportional sampler at 60 min intervals collected 24-h composite influent wastewater samples to ensure representative samples. The sampling interval (60 min) was fixed by the WWTPs according to the Spanish legislation [21]. Ort et al. [22] and Castiglioni et al. [10] established that the best practice sampling method is to use a continuous flow-proportional sampler. This was however not possible at the WWTPs we investigated and only time-proportional sampling was possible at 60 min intervals. Due to the size of the catchment populations investigated, it

is still expected that the uncertainty based on the sampling would be less than 10% and thus smaller than the analytical uncertainty.

Samples were taken during 7 consecutive days, starting on March 4, 2015. All samples were collected in triplicate in 1 L polyethylene terephthalate (PET) amber bottles and sent to the laboratory in a cooler. Once there, they were kept frozen at −20 °C until extraction, minimizing the degradation of the studied compounds. The WWTPs recorded the flow rate daily, Quart-Benàger analyzed BOD, COD, total N and total P daily except on weekends (corresponding to Friday and Saturday, as samples were analyzed the day after). At Pinedo I and II, COD was recorded daily (including weekends), but the other parameters are only measured once a week.

Regarding the stability of biomarkers in the sewer network, the three WWTPs are located at the end of the catchment. In the case of Quart-Benàger, the travel distance of the sewage was from 1 to 25 km and the mean and maximum residence times are 10 and 16 h, respectively, and in the case of Pinedo I and II, the travel distance was from 0 to 15 km and the mean and maximum residence times 7 and 12 h. Laboratory assays were carried out looking at the biomarker stability under these conditions.

2.3. SPE extraction

An off-line solid phase extraction (SPE) reported by Andrés-Costa et al. [23] was used for the pre-concentration of selected analytes in wastewater samples. Internal standards were added to 250 mL of WWTP influent samples to obtain a final concentration of 25 μg/L in the extract (that means a concentration in water samples of 100 ng/L) then, the samples were vacuum filtered on glass fiber filters (GA-55, 90 mm, Advantec) to remove particles, and loaded onto Strata-X 33 μm Polymeric Reversed Phase (Phenomenex) SPE cartridges preconditioned with MeOH (6 mL) and Milli-Q water (6 mL). After sample percolation, cartridges were left to dry for 15 min under vacuum. Then, analytes were eluted with 6 mL of MeOH, and eluates were evaporated to dryness under a gentle stream of nitrogen gas at 40 °C. Extracts were reconstituted in 1 mL of MeOH—H_2O (10:90, v/v), sonicated for 1 min and kept at −20 °C until analysis within 15 days.

2.4. Dilute and shoot method

A volume of 10 mL of wastewater samples were spiked with 50 μL of 10 μg/mL of internal standard to obtain a final concentration of 50 μg/L. Then, samples were diluted with distilled water (50:50) and centrifuged for 3 min at 4000 rpm. The supernatants were transferred to 2 mL amber vials stored at −20 °C and analyzed within 15 days.

2.5. LC–MS/MS analysis, quantification and quality control

Samples were analyzed for the target compounds using a liquid chromatograph Agilent 1260 UHPLC coupled with a triple Quadrupole spectrometer (QqQ) Agilent 6410 (Waldbronn, Germany) (conditions are listed in the Supplementary content text and Tables S7.3–S7.5 and chromatograms obtained in Fig. S7.2).

Quantitative analysis of biomarkers was performed by external calibration, but three isotopically labelled compounds, i.e. CET-d3 and morphine-d3 for positive ionization mode, and THCOOH-d3 for negative ionization mode, were added to all the samples before the extraction as control standards at concentrations reported in Sections 2.3. and 2.4. depending on the analytical method. The observation of the internal standard area provided information on problems arising during the acquisition of samples or the existence of matrix effects.

Furthermore, a strict quality control was followed in order to ensure accurate results. Before and after each sampling batch (between 25 and 30 samples), calibration curves were injected. After every 15th samples, one instrumental and one procedural blank as well as one positive control (wastewater spiked at 100 ng/L prior to extraction) and one matrix matched standard (wastewater extract spiked at 100 ng/L) were analyzed to serve as quality control. Spiked and non-spiked samples were analyzed in triplicate. Quantitative analysis of illicit drugs was carried out using internal standard calibration as previously reported [23]. In every batch of analysis, several blank samples, calibration standards at several concentration levels, matrix-matched standards and control samples were analyzed as quality assurance, finding no contamination in blanks and good agreement between the concentrations found in calibration standards and their true content.

2.6. Stability study

Stability of biomarkers in the sewer system and during transport and storage is a crucial question. The stability was checked through laboratory studies in which samples were stored at room temperature (average value 26 °C), 4 °C and −20 °C. The samples stored at 26 °C were analyzed at t 0, 2, 4, 6, 12, 18, 24 and 36 h. The samples stored at −4 °C were analyzed at t 0, 12, 24, 36 and 48 h. The samples at −20 °C were analyzed weekly for 4 weeks (maximum time of storage in the laboratory). Sample extracts were also tested weekly for three months and no degradation was observed.

2.7. Consumption, metabolism and excretion data

The population size was estimated according to the following equation:

$$Population\,(inh.) = \frac{Cbk \times F \times E_R}{DDD}$$

where *Cbk* is the concentration of biomarker (ng/L), *F* is the flow rate (L/day), E_R is the excretion rate (adimensional) and *DDD* is the defined daily dose (mg) per 1000 inh. To obtain accurate estimations of population, consumption data and excretion rates are needed for each analyte. Data used are presented in Table 7.1. Con-sumption was obtained through the Spanish Ministry of Health (AEMPS) [24] and the ATC/DDD index of the World Health Organiza-tion (WHO) [25], except for codeine [26], and excretion factors were obtained from several sources [27,28,29,30,31,32]. For caffeine and acesulfame, excretion per day per 1000 inh was calculated with consumption estimations [33,34], and excretion factors [35,36]. For nicotine metabolites, cigarette consumption was estimated from tobacco sales in Valencia [37] and census data [38] and excre-tion factors were calculated with percentages of excretion of the metabolites in urine and molecular mass ratios [39]. For 5-HIAA, the daily average excretion per 1000 inh is 3.44 mg [40].

For creatinine, excretion was calculated using three different equations [41,42,43], with average weight (in kg) and age obtained from the IVIE database [44] (equations are outlined in the Supplementary content). Average estimations obtained were 1322, 1425 and 1373 mg/inh/day for the Ix, Fotheringham and Walser equations, respectively (detailed estimations obtained are shown in Table S7.6). As the estimations were similar, the average of the three methods (1373 mg/inh/day) was used.

Table 7.1
Daily excretion of pharmaceuticals (hydrochlorothiazide (HCTZ), carbamazepine (CBZ), codeine, naproxen, salicylic acid (SA) and atenolol), caffeine, acesulfame and nicotine metabolites (cotinine and hydroxycotinine (OH—COT)) and data used for their estimation.

	Consumption(DDD/1000 inh)	DDD (g)	Excretion factor	Excretion (g/1000 inh)
HCTZ	10.82[a]	0.025	0.95	0.257
CBZ	1.23[a]	1.00	0.01	0.012
Codeine	235.00[a]	0.100	0.04	1.01
Naproxen	7.20[a]	0.500	0.01	0.040
SA	33.09[a]	3.00	0.17	17.07
Atenolol	9.06[a]	0.035	0.90	0.286
Caffeine	0.264[b]		0.03	7.92
Acesulfame	0.057[b]		1.00	57.00
Cotinine	3.63[c]//2.90[d]		0.14	0.429
OH—COT			0.53	1.81

inh: inhabitant; DDD: Defined Daily Doses.
[a] Consumption expressed in DDD/1000 inh.
[b] Consumption expressed in g/day inh.
[c] Cigarette consumption expressed in cig/inh day.
[d] Nicotine consumption expressed in g/1000 inh day.

2.8. Population-normalized drug consumption

The back-calculation was carried out according to the equation:

$$Drug\ \ use = \frac{(Ctr \times F) \times Cf}{Population}$$

where *Drug use* is measured in mg/day/1000 inh, *Ctr* (ng/L) is the concentration of target residue in the wastewater sample, *F* (L/day) is the flow rate of wastewater, and *Cf* is a correction factor obtained considering excretion rates of drug residues and the molecular mass ratio of parent drug/metabolite Zuccato et al. [45].

2.9. Statistical analysis

Statistical analysis was made with IBM SPSS Statistics 22 and Microsoft Office Excel 2013. For the One-Way analysis of variance (ANOVA) test, it was decided that P-values higher than 0.05 indicated that there was no statistical difference between the two averages compared.

3. Results and discussion

3.1. Validation of analytical procedure

SPE followed by LC–MS/MS could detect all the selected analytes with the exception of 5-HIAA and creatinine. Due to the minimal extraction of 5-HIAA and creatinine in the SPE method, samples were analyzed using a dilute and shoot approach, with only a step of centrifugation. Seven compounds were also detected by dilute and shoot LC–MS/MS. The main reason for using SPE is the lower concentrations of other biomarkers in influents, which need isolation and concentration to achieve the required sensitivity.

All performance results of the LC–MS/MS analytical methods are outlined in Table 7.2. Validation was carried out analyzing five replicates of each sample in a mixture with equal volumes of the three WWTPs influents. Curves were linear with correlation coefficients (R^2) ranging from 0.9967 to 0.9996. Using SPE, LOD were between 3 and 309 ng/L, and LOQ between 9 and 1030 ng/L. Recoveries ranged from 31 to 143% with the exception of 5-HIAA and creatinine. Recoveries <75% are low but it should be taken into account that these are absolute recoveries. In further improvements of the method, these recoveries could be corrected using the isotopically labelled internal standard of each analyte. The matrix complexity should also be considered. Matrix effect was always suppression ranging from 2 to 50% and it was corrected by the use of internal standards in the case of the illicit drugs. For the other compounds, matrix matched standards at 1 μg/L were used. The overall process efficiency was low for some of the target compounds.

The correction of the matrix effects was not carried out using matrix matched calibration but only one matrix matched standard because the concentration of these compounds in the influent water is high. Then, establishing linearity in matrix matched standards at low concentration is difficult. The final result was recovery corrected, this may be an unorthodox approach but as the validation process was carried out in the same samples where the analytes were measured, the approach is able to capture the impact of sample characteristics on the results. Intra-day and inter-day precisions were satisfactory, ranging from 3.5 to 8.7% and from 4.1 to 9.6%, respectively.

Performance of the dilute and shoot method is also shown in Table 7.2. LOD range from 500 to 15,000 ng/L, and LOQ from 2000 to 50,000 ng/L. Matrix effects ranged from 11 to 65% and were corrected using a matrix matched standard spiked at 25 μg/L. Intra-day coefficient of variation (CV) is lower than 10% for all analytes, and inter-day CV were between 4.2 and 11.8%. The direct injection of water samples after centrifugation (without dilution) was also tested but matrix effects were not acceptable and prevented the determination, particularly, of 5-HIAA. The dilution (50:50) with distilled water provided proper validation parameters for 9 of the 12 studied biomarkers.

3.2. Occurrence of urine biomarkers in untreated wastewater

The range and average concentrations in wastewater samples, calculated where possible by both methods, are shown in Table 7.3. SPE values were corrected with the recoveries obtained for the calibration standards included in every batch of analysis. Only cre-atinine concentrations calculated by SPE extraction were markedly lower. From now on, the concentrations of 5-HIAA, atenolol, caf-feine, codeine, cotinine, creatinine and OH COT will refer to the ones obtained by dilute and shoot, and those of acesulfame, CBZ, HCTZ, naproxen and SA will be the ones obtained with the SPE method (see Table S7.7 Supplementary content for derived calculated loads in g/day).

Acesulfame concentrations ranged from 3.30 to 30.13 μg/L in agreement with those found in Germany (42.00 μg/L) [46] and Australia (ca. 40 μg/L) [16]. Atenolol values were in the range of 15.16–90.11 μg/L, higher than those measured in Germany (0.07 μg/L) [46]. Caffeine concentrations were 21.93–98.73 μg/L, similar to those reported in Seville, Spain (30.2 μg/L in winter and 48.5 μg/L in summer), United States (11.5–120 μg/L), and Italy (17.6–67.6 μg/L) [14,20,47] and higher than those found in China (3.4–6.6 μg/L) [48].

Table 7.2
Results of the validation of the analytical methods carried out in a mixture with equal volumes of the three WWTPs influents spiked with a mixture of 16 substances.

	Linearity(R^2)[a]	LOD[b] (μg/L)	LOQ[c] (μg/L)	Matrix Effect[d] (%)	Recovery[e] (%)	Intra-day[f] CV (%)	Inter-day[g] CV (%)
SPE method							
Acesulfame	0.9969	0.063	0.208	27	31	6.2	7.3
Atenolol	0.9994	0.309	1.03	28	50	7.7	9.6
Bufotenine	0.9990	0.004	0.013	32	86	5.3	4.9
Caffeine	0.9996	0.022	0.073	22	38	4.1	5.9
CBZ	0.9991	0.003	0.009	12	32	8.7	8.5
CET	0.9991	0.004	0.013	22	88	3.5	4.1
Codeine	0.9982	0.024	0.081	15	36	7.1	7.6
Cotinine	0.9996	0.021	0.069	28	29	4.7	5.5
HCTZ	0.9995	0.003	0.009	40	143	4.2	5.3
Morphine	0.9991	0.004	0.013	25	101	7.9	8.8
Naproxen	0.9993	0.025	0.082	50	43	3.7	6.1
OH—COT	0.9992	0.008	0.027	17	52	4.1	5.7
SA	0.9989	0.093	0.309	15	32	8.2	8.9
THC—COOH	0.9990	0.014	0.046	25	61	5.3	6.1
Dilute and shoot method							
5-HIAA	0.9975	1.00	3.00	65	–	5.3	10.1
Atenolol	0.9994	3.00	10.00	45	–	9.3	11.8
Caffeine	0.9996	1.00	3.00	35	–	2.9	4.2
CBZ	0.9991	15.00	50.00	11	–	9.8	11.3
Codeine	0.9982	0.50	2.00	18	–	6.4	7.1
Cotinine	0.9996	0.50	2.00	32	–	7.5	9.1
Creatinine	0.9967	0.50	2.00	50	–	4.3	8.9
Naproxen	0.9993	1.00	3.00	28	–	8.1	9.7
OH—COT	0.9990	0.50	2.00	50	–	4.4	10.9

[a] Calibration curves were constructed with 18 points at 6 different levels, from 2 to 2000 μg/L for population biomarkers.
[b] LOD were estimated based on signal-to-noise (S/N) ratio of 3 and correcting the values with recovery data for SPE LC–MS/MS method.
[c] LOQ were estimated based on S/N = 10, correcting the values with recovery data for SPE LC–MS/MS method.
[d] Calculated for SPE comparing the peak area of the SPE extracts spiked with 1 μg/L after subtracting the area of the peak in the non-spiked extract with that obtained for the standard at the same concentration prepared in MeOH:H_2O (10:90). For "diluted and shoot", were calculated comparing the water spiked at 50 μg/L after subtracting the non-spiked with that obtained for the standard at the same concentration prepared in MeOH:H_2O (10:90). The experiments were carried out in quintuplicate.
[e] Calculated from wastewater samples spiked at a 1 μg/L level and extracted. Experiments were always carried out in quintuplicate. The recoveries were established comparing the peak area with that obtained for the matrix matched standard at the same concentration. Non-spiked samples were also analyzed to check the amount in samples. Recoveries reported were absolute recoveries (not corrected by any internal standard).
[f] Replicates injected in the same day ($n = 5$). For SPE LC–MS/MS include extraction.
[g] Replicates injected on different days ($n = 5$). For SPE LC–MS/MS include extraction.

CBZ values were lower than those reported for Germany (0.66 μg/L), Portugal (0.47 μg/L), China (0.11 μg/L) and Spain (0.07–0.17 μg/mL) [27,46,47,48] but close to those of Southwest China (0.01 μg/L) [49]. This variability in concentrations may be explained by the fact that, since CBZ was introduced into the market, various alternatives for this antiepileptic drug have become available, depending on their prescription in the country. Codeine concentrations were between 0.28 and 0.91 μg/L, higher than those measured in Catalonia, Spain (0.06–0.12 and 0.02–0.12 μg/L) but similar to the ones found in Miami, United States (0.01–0.98 μg/L) [50,51,52].

Cotinine concentrations ranged between 1.10–4.13 μg/L, similar to those found in Portugal (1.13–3.50 μg/L), Italy (0.65–3.12 μg/L), United States (0.13–2.70 μg/L) and Spain (0.3–1.9) [14,20,53,54]. Creatinine concentrations were lower than those found in the United States (between 220.00 and 1500.00 μg/L) [14]. This is probably because preliminary data indicate that creatinine is quite unstable in wastewater sample. [18,55]. This hypothesis correlates and seems to explain also, why concentrations obtained by the dilute and shoot approach are higher than those measured with SPE.

HCTZ was in our samples at a concentration between 17.57 and 37.49 μg/L, higher than the ones reported in Australia (ca. 1 μg/L) [16]. Naproxen concentration was between 2.61 and 4.36 μg/L, similar to the ones measured in Spain (4.28 and 8.65 μg/L) and Australia (ca. 1.5 μg/L) [16,47]. OH—COT values obtained were from 1.09 to 3.60 μg/L for the SPE method and from 1.89 to 3.43 μg/L for the method without extraction. Similar data was obtained in Spain (1.0–3.3 μg/L) and Italy (2.14–7.00 μg/L) [20,54]. SA concentrations were measured between 9.68 and 27.28 μg/L, in good agreement with those found in Spain (14.3–94.3 μg/L) and Australia (ca. 35 μg/L) [16,47].

These data support that many aspects may influence the concentration differences between cities and countries such as higher per capita flow, differences in consumption, differences in amount prescribed, different residence times in the sewer and types of sewers which may impact the degradation and so on [55]. Further research will help to quantify and explain them, and to establish uncertainty due to these factors.

3.3. Stability study

As previously reported, most of the selected biomarkers were stable under laboratory conditions [14,18,20]. Freezing is the convenient method for sample preservation between collection and extraction. All the compounds, biomarkers and drugs of abuse, were stable at −20 °C for up to four weeks (maximum time that samples were stored in this study).

Samples stored at room temperature and at 4 °C showed also appropriate stability for all the biomarkers with the exception of creatinine that degrades an average of 65% at 24 h in raw influent at room temperature and up to 50% at 4 °C. According to Chen et al. [18], creatinine decomposed virtually completely within 24 h in untreated wastewater. In our study, the decomposition was not complete but results also indicates that creatinine could be too unstable to be used as biomarker. Chen et al. [18] also found that cotinine and 5-HIAA were stable in raw wastewater over long periods of time. The stability in laboratory conditions of cotinine and caffeine was appropriate and completely agree with results recently reported by Senta et al. [20].

Table 7.3
Concentrations found in wastewater samples.

	Quart-Benàger		Pinedo I		Pinedo II	
	SPE(μg/L)	Dilute and shoot (μg/L)	SPE(μg/L)	Dilute and shoot (μg/L)	SPE(μg/L)	Dilute and shoot (μg/L)
BIOMARKERS						
5-HIAA[a]		5.53–10.54		7.93–14.31		8.80–10.60
		7.91		**11.29**		**9.52**
Acesulfame[b]	9.59–30.13		3.30–11.78		3.60–8.64	
	16.08		**7.98**		**5.60**	
Atenolol[a]	15.16–32.70	15.21–90.11	14.34–46.67	29.55–62.16	17.52–37.50	22.79–36.78
	20.27	**49.44**	**27.28**	**38.54**	**26.36**	**30.03**
Caffeine[b]	30.08–53.80	49.04–98.73	24.72–29.61	32.97–40.87	21.93–27.49	33.70–38.11
	41.13	**77.86**	**27.49**	**36.34**	**24.62**	**36.12**
CBZ[b]	0.010–0.030		0.006–0.016		0.002–0.013	
	0.017		**0.012**		**0.007**	
Codeine[a]	0.278–0.555	0.331–0.905	0.297–0.389	0.304–0.511	0.316–0.529	0.406–0.576
	0.362	**0.533**	**0.338**	**0.389**	**0.406**	**0.475**
Cotinine[a]	1.10–2.10	1.63–4.13	1.44–1.89	1.88–2.20	1.38–1.87	1.52–2.46
	1.62	**2.79**	**1.65**	**2.10**	**1.59**	**1.86**
Creatinine[a]	0.298–3.27	0.49–2.72	0.200–0.415	0.775–2.22	0.308–0.611	1.48–2.31
	1.08	**1.88**	**0.314**	**1.65**	**0.466**	**1.88**
HCTZ[b]	20.17–30.09		17.73–37.49		17.57–36.35	
	25.61		**26.43**		**29.68**	
Naproxen[b]	2.61–4.36		2.67–3.07		2.97–3.58	
	3.12		**2.85**		**3.24**	
OH—COT[a]	1.09–2.57	1.89–3.43	1.69–3.60	2.12–2.56	1.67–3.01	1.99–2.32
	1.98	**2.63**	**2.88**	**2.29**	**2.46**	**2.17**
SA[b]	13.35–23.57		9.68–25.42		10.23–27.28	
	17.43		**19.87**		**16.51**	
ILLICIT DRUGS						
Bufotenine	0.016–0.057		0.014–0.040		0.033–0.104	
	0.030		**0.025**		**0.067**	
CET	0.015–0.063		0.014–0.039		0.013–0.045	
	0.034		**0.025**		**0.027**	
Morphine	0.075–0.217		0.068–0.174		0.069–0.183	
	0.165		**0.124**		**0.129**	
THC—COOH	0.213–0.360		0.209–0.331		0.210–0.360	
	0.272		**0.262**		**0.273**	

Values in bold are the averages.
[a] Biomarkers quantified using the dilute and shoot method.
[b] Biomarkers quantified using the SPE method.

It should be noted that these stability studies were carried out in the laboratory, and the real case scenario in sewer pipes add various microbial, chemical and physicochemical processes that could produce additional degradation. However, these stability findings were confirmed in a recent study on in-sewer transformation on 43 pharmaceuticals by Jelic et al. [56]. For most compounds (including naproxen and atenolol in common with our study), the average removal, calculated pair-wise (influent-effluent, for each sampling day) and then averaged, ranged from −10 to 10%, which could be considered negligible when compared with the overall uncertainty associated with the concentration values.

3.4. Estimating population size

3.4.1. Hydrochemical parameters

Population size was first estimated according to the hydrochemical parameters. The number of inhabitants was established dividing BOD by 60, COD by 128, N by 10 and P by 1.7 as estimations of the amount produced by individuals (exhaustive population size calculations are in Table S7.8 and Fig. S7.3 and S7.4 of the Supplemen-tary content). For Pinedo I and II, COD was the only parameter determined daily. The others were determined only once a week on Tuesday. Population calculated according to COD showed some daily variations (minimum on Saturdays), and agree with popula-tion estimated with N and BOD. However, population estimated in Pinedo I with P is 5 times higher than those obtained by COD, N and BOD. On the contrary, population estimated for Pinedo II using P was half that when compared to results obtained by COD, N and BOD. As there was only one data point measured within the week, further interpretation of this result would be limited and is therefore not discussed. Both WWTPs show a drastic decrease in population based on COD on Saturday and Sunday, of approximately 100,000 in Pinedo I and 500,000 inh in Pinedo II that could be due to commuting patterns. Since these two WWTPs only treat urban water, the estimations based on hydrochemical parameters must provide an appropriate estimation of the population. In Quart-Benàger, the estimation based on BOD is the highest and on P the lowest, with an average of 206,000 and 152,050 inh, respectively. The trend for all parameters showed a lower population on Sunday which may be indicative of a transient population where workers in Valencia reside elsewhere. This could easily be an erroneous conclusion, as approximately 60% of the wastewater of Quart-Benàger comes from industrial sources, and this decrease in the hydrochemical parameters could be due to the lower industrial activity during weekends. However, to complete the picture both hypothesis could be true. According to the 2010 census the metropolitan area of Valencia had a population of 1,542,233 inh (including Valencia city) [57]. The calculated population according to the COD (recorded daily in all the WWTPs) would be 1,645,197 which is only a slight overestimation. The design capacity (Table S7.2) accounts for a popu-lation served of 1,508,972, then, the differences are not statistically significant ($p > 0.05$).

In this study, only a week of samples is covered. Then, the weekly variations may not be true since these parameters could only be affected this particularly week. However, these WWTPs have also been monitored during previous years (2011–2013) and the decrease in population during the weekend was also observed [23]. Therefore, the trend established in behavior of these hydro-

chemical parameters and the population size estimation associated to them appears to be confirmed.

3.4.2. Human urine-biomarkers

Compared to the hydrochemical parameters (COD, BOD, N and P), atenolol, HCTZ and naproxen always estimated higher populations and SA, acesulfame CBZ, codeine, creatinine and OH—COT lower. Only 5-HIAA, cotinine and caffeine reveal possibilities to be used in some cases as biomarkers. This was already suggested in previous attempts [5,14,15,18,19]. Fig. 7.1 shows the box and whiskers plots for estimated populations using hydrochemical parameters and human biomarkers. Three outliers were found, one for Pinedo I (atenolol, Sunday) and two for Pinedo II (OH—COT and naproxen, Wednesday). Individual results for biomarkers are listed in Table S7.9 in the Supplementary content and summarized in Fig. S7.5 to S7.7 in the Supplementary content. Back-calculation of the con-sumption of a compound relies on specific correction factors, which take into account the urinary metabolism of a substance. In this study, the excretion rates used were obtained using the few avail-able studies. Therefore, the possibility remains that the excretion values used were imprecise, leading to an inaccurate estimation of population size. This should be considered as an added uncer-tainty. Some of these biomarkers are suggested to be used by a more elderly population. However, this has been corrected in the calculation of DDD/1000 inh. Furthermore, the average age in Spain (42.5 years) is similar to that of Valencia metropolitan area (42.2 years), so the uncertainty derived from a younger or older popula-tion should be minimal.

Inappropriate creatinine and OH—COT estimations can be attributed to their instability in wastewater [17,18]. The estimations based on pharmaceuticals were calculated with the amounts of these products prescribed in Spain, not taking into account over-the-counter sales or local differences. Furthermore, the only data available was for 2006 in some cases. Since then, consumption patterns could have changed. For acesulfame, the estimation was based on Portuguese consumption, as this kind of data is not available for Spain. To obtain reliable estimations based on these parameters, updated consumption studies would be needed [5]. However, concentrations of HTCZ, naproxen, carbamazepine and codeine were consistent over the weekdays (without population commuting). Therefore, they could be appropriate biomarkers of the population size with the appropriate data about consumption or the appropriate modeling to adjust populations [16,17,18,19].

Estimations made with daily loads of cotinine and caffeine are similar to the stated population in Pinedo I and II. In the case of Pinedo I, inh calculated from 5-HIAA concentrations also seem to be accurate. The ANOVA test made for the three WWTP and the weekly averaged population, (Supplementary content, Table S7.10), showed no statistically significant differences between the esti-mations made through cotinine, caffeine and COD for Pinedo I, between those made with caffeine and cotinine or COD and 5-HIAA for Pinedo II and between those through cotinine, BOD, COD and total N in Quart-Benàger. In this last WWTP, caffeine appears to overestimate population during the weekdays and showed an abrupt decrease in population during the weekend. Although due to commuting patterns, the number of inh may be higher on weekdays, it is unlikely that the population doubles its size. This could also be explained by a higher intake of caffeine-containing beverages in working days (also difficult to confirm) or by a carbonated cola soft-drink bottling plant that discharges to this WWTP. During the week, workers may clean tanks and bottles, disposing the effluents into the sewage system and increasing the load of caffeine. During weekends, the cleaning process may stop, explaining the decrease in caffeine concentration, and making the estimation of inh based on caffeine more realistic.

The ANOVA test was performed comparing the daily estimations instead of the averages (Supplementary content, Table S7.11). For Pinedo I and II, there was no statistical difference between the estimations made with cotinine and caffeine, and in Pinedo I, there was also no difference between those made with COD and 5-HIAA (at least for a couple of days) whereas in Pinedo II only on Saturday there were no differences between COD and cotinine. In Quart-Benàger there was no statistical difference between the population estimated with cotinine and COD, BOD and N (except on Sunday). In this WWTP, differences were observed on Saturday and Monday between cotinine and caffeine, and on Sunday, between caffeine, COD, BOD, N and P suggesting to the hypothesis that anomalous results on caffeine are due to the existence of the cola soft-drinks bottling plant.

3.5. Illicit drugs occurrence and estimation of its consumption

In order to demonstrate the viability of the population estimated using these biomarkers, the consumption of THC (based on THC—COOH), cocaine (based on CET), bufotenine and heroin (based on morphine) was calculated. Using the molecular mass ratio between the illicit drug and its measured metabolite (if the case) and the excretion rate, the amount of drugs consumed by the inh served by each WWTP was calculated. Then, g/1000 inh were estimated with the population obtained by the hydrochemical parameters and biomarkers (Fig. S7.8 to S7.19 of the Supplementary content).

On the occurrence of illicit drugs, concentrations of CET (0.03 μg/L) were lower than those found in several Italian cities (0.03–0.06 μg/L), but similar to the ones measured in Chicago (0.02 μg/L) [58]. Morphine results (0.12–0.17 μg/L) were higher than those reported in Catalonia, Spain (0.59 μg/L) [51]. THC—COOH concentrations (0.26–0.27 μg/L) were lower than the ones found in France (0.27–1.16 μg/L) [4], but higher than the ones found in Catalonia, Spain (0.02–0.04 μg/L) [51].

Consumption averages are summarized in Table 7.4. The results show that cannabis, heroin and bufotenine consumption remained relatively stable during the week, while cocaine consumption dou-bled on weekends. Consumption of cannabis, cocaine, heroin and bufotenine was calculated for each WWTP and day, showing similar consumption levels in Pinedo I and II, and a lower one in Quart-Benàger, except in the case of bufotenine, where the normalized loads were closer in Quart-Benàger and Pinedo I and estimates three times higher for Pinedo II.

For Quart-Benàger, consumption trends are similar with all population estimations, except for caffeine. For Pinedo I, data obtained with caffeine, cotinine and WWTP design capacity estimations are in good agreement, while the ones made with 5-HIAA loads and COD show a different trend, with a higher consumption during weekends. For Pinedo II, results were similar, with the data estimated with COD being slightly different from the rest. In Pinedo I and II, there was a strong correlation between the averages with the estimations made with caffeine and nicotine loads, and the WWTP design capacity, with a difference of only 0.3 and 1 g/1000 inh for Pinedo I and II, respectively. The average amount consumed in Pinedo I and II is similar, but higher than in Quart-Benàger.

These estimations of the drug consumption have some limitation due to the metabolites used. Many of them have been pointed out by the Sewage Analysis CORe group Europe (SCORE) [59], others are also highlighted in a number of reviews [60,61]. The THC—COOH loads in sewers based on urine excretion data may be wrong as the bulk of THC is excreted through feces, which may transform to THC—COOH leading to overestimation of THC consumption. Morphine itself may also enter the wastewater, as do other drugs/pharmaceuticals which metabolize to morphine, and as such morphine as a marker for heroin use may overestimate

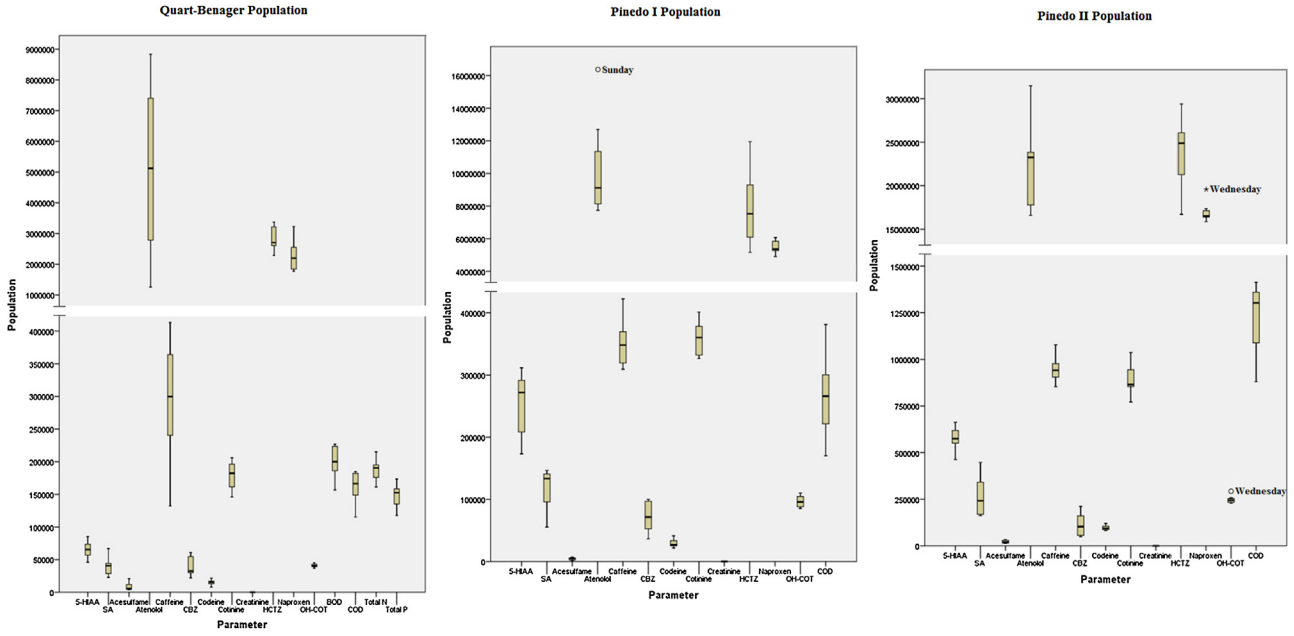

Fig. 7.1. Box and whiskers plots for population estimated with urine biomarkers and hydrochemical parameters.

Table 7.4
Average cannabis (Cann), cocaine (Coc), heroin (Her) and bufotenine (Buf) consumption during a week normalized with different population estimations.

	Quart-Benàger				Pinedo I				Pinedo II			
	Doses/1000 inh day				Doses/1000 inh day				Doses/1000 inh day			
	Cann	Coc	Her	Buf	Cann	Coc	Her	Buf	Cann	Coc	Her	Buf
Caffeine	35	5	18	6	70	8	28	10	73	8	29	28
Cotinine	54	7	27	9	68	7	27	10	78	8	31	31
COD	59	8	30	10	94	11	37	13	57	7	23	22
BOD	47	6	24	8								
WWTPdesign capacity.	44	6	22	8	69	7	28	10	73	8	29	29
Total N	51	7	26	9								
Total P	64	9	32	11								
5-HIAA					101	11	39	14				

actual heroin consumption [61]. Furthermore, cocaine is commonly estimated using benzoylecgonine which is the most stable metabolite of cocaine and it should be noted that cocaethylene is only a metabolite of cocaine when consumed with alcohol. However, the recreational mixing of alcohol and drugs has increased dramatically over the past decades, therefore, we consider it an acceptable calculation.

The estimated consumed loads of the illicit drugs in this study have been compared against other drug consumption estimates for Valencia based on previously published data [23]. Cannabis consumption was always higher in Pinedo I (4,034.4, 4,163.2 and 12,422.5 mg/day/1000 inh in 2011, 2012 and 2013 respectively) than in Pinedo II (1,935.0, 1,579.6 and 10,591.0 mg/day/1000 inh) or Quart-Benàger (1,880.0, 1,743.4 and 9,419.8 mg/day/1000 inh) and underwent a large increase in 2013 in the three WWTPs. The data reported here on 2015 (See Figs. S7.8–S7.10) shows a sta-ble trend in the consumption since the results are very similar to 2013. The same happens with cocaine consumption even though in this case the cocaine estimation was carried out with a dif-ferent biomarker. The estimated COC consumption in 2011 was 1,641.3, 1,181.7, 1,332.6 mg/day/1000 inh in Pinedo I, Pinedo II and Quart-Benàger, respectively, which decreased to 1,191.3, 972.8 and 1,034.9 mg/day/1000 inh respectively in 2013. Data in 2015 (Figs. S7.11–S7.13), even though calculated using a different metabo-lite indicate a decreasing trend. The consumption of heroin through morphine was not estimated because of the problem with the overestimation, and the bufotenine, being as a new psychoactive substance, was not covered in our previous study.

4. Conclusions

All analytes studied as possible indicators of the human population were present in wastewater samples. By any of the two analytical methods developed, intra and inter-day variations were lower than 12% for all analytes, with LOQ ranging from 9 to 1030 ng/L for the SPE method and from 2 to 50 μg/L without extraction. However, the SPE method provided low recoveries of some urine biomarkers and the dilute and shoot approach showed high LOQs for some others. Among the biomarkers of human population studied, population estimations made with daily loads of caffeine, cotinine, and in one WWTP, also with 5-HIAA, were the most accurate and comparable to the census data and hydrochemical parameters. In the case of Pinedo I, calculations made with COD and 5-HIAA were not statistically different. Although the most useful biomarkers were cotinine and caffeine, HCTZ, naproxen, codeine and CBZ have good prospects if the appropriate data are available. The use of biomarkers to estimate population should be evaluated very carefully, because even in the case of caffeine, special cases as the presence of cola soft-drink bottling factories can distort the results. However, they can be an invaluable tool to reduce uncertainty in population size served by the WWTP.

Acknowledgement

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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.jhazmat.2016.05.079.

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SUPPLEMENTARY CONTENT

Use of Urine Biomarkers to Estimate Population Size in Sewage Epidemiology Illicit drugs consumption in Valencia Metropolitan area as study case.

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Introduction

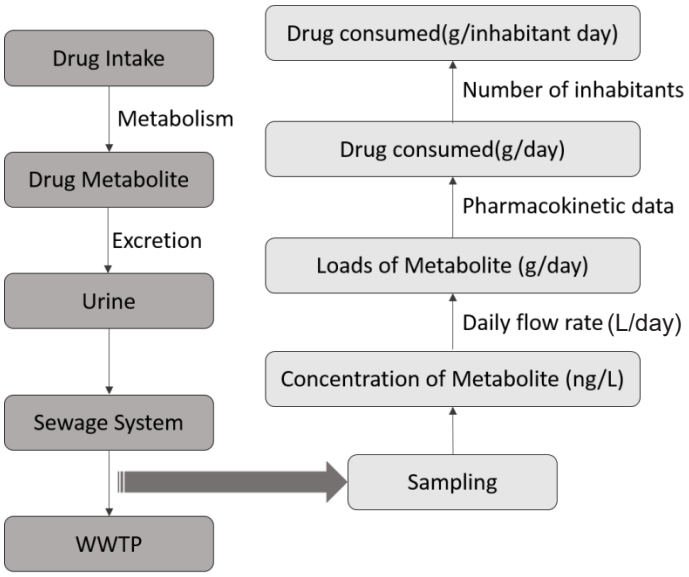


Fig. S7.1. Schematic overview of WBE applied to the case of illicit drugs.

Experimental

Chemicals and reagents

All analytes studied, with their formula, molecular weight and CAS number are shown in Table S1.

- Hydrochlorothiazide (HCTZ)

HCTZ is a diuretic, used in congestive heart failure, hypertension and to decrease fluid buildup. HCTZ is usually taken orally, is available as a generic drug and it's not expensive. This drug has a 70% of bioavailability in humans, and it's more than 95% eliminated unchanged in urine (Beermann et al. 1976). Due to it being a widely prescribed substance, it was decided to analyze it in WW samples.

- Caffeine

Caffeine is a central nervous system stimulant of the methylxanthine class of psychoactive drugs. It occurs naturally in many plants, and is mostly derived from coffee and cocoa beans, and tea leaves. Caffeine is widely used in food and drinks, as a dietary supplement and in medication. It’s one of the most consumed food ingredients worldwide, with coffee and tea the most prominent sources in the diet. 3% of the ingested caffeine is excreted unchanged in urine (Mandel, 2002). Taking into account that coffee and tea are two of the most commonly consumed beverages worldwide, only after water (Choi and Curhan, 2007), the analysis of caffeine in WW could provide us with a good population estimate.

- Carbamazepine (CBZ)

CBZ is an anticonvulsant drug which is widely used for the treatment of epileptic seizures, trigeminal neuralgia and some psychiatric disorders, such as bipolar disorder. Although these are not extremely common diseases, considerable amounts of this pharmaceutical are consumed due to the high daily dose of between 800 and 1200 mg (Epilepsy Society, 2014). In terms of its pharmacokinetic properties, only 1% of the parent compound is excreted in urine (Bahlmann et al., 2014).

- 5-Hydroxyindole-3-acetic acid (5-HIAA)

5-HIAA is the primary metabolite of serotonin. Serotonin is a biogenic amine, derived from tryptophan that acts as a neurotransmitter in the central and peripheral nervous systems, controlling many brain functions and regulating blood pressure and muscle contraction. The average daily excretion of 5-HIAA in urine is 3.44 mg, but this quantity can be higher in people with carcinoid syndrome, celiac disease or by the consumption of food or drugs containing serotonin (Joy et al., 2008).

- Codeine

Codeine is an opiate analgesic used to relieve mild to moderate pain. It's also used, usually combined with other medications, to reduce coughing. It has a 90% of bioavailability, taken orally, and 4.3% is excreted unchanged in urine (Yue et al., 1989).

- Acesulfame K

Acesulfame is an artificial sweetener and flavor enhancer, also known under the additive code E950. It's 200 times sweeter than sugar, and it's used as a food additive, in carbonated drinks and even in pharmaceuticals products. This compound passes unmetabolized through the body, and is rapidly excreted in urine (Renwick, 1986).

- Creatinine

Creatinine is a breakdown product of creatine, a nitrogenous organic acid that helps to supply energy to cells, mainly muscle. Creatinine is removed from the body by the kidneys, and is often used to normalize the concentration of other compounds when analyzing urine samples.

Every day, approximately 1.5% of muscle creatine is transformed to creatinine. High concentration of creatinine in urine can appear in a heavy meat-based diet, or also due to kidney failures (Hellerstein et al., 2006). Seeing as it is a compound always found in urine at relatively high

concentrations, and that it suffers next to no intra-individual variations, its analysis in WW samples could provide us with a very good estimation of the population served by each WWTP.

- Naproxen

Naproxen is a non-steroidal antiinflamatory drug (NSAID), employed commonly to reduce inflammation, pain and fever, blocking the enzymes that produce prostaglandins. It has a 95% bioavailability when orally administered, and the parent compound accounts for 1.1% of the dose recovered in urine (Vree et al., 1993). Due to its wide use, it was decided to analyze it in WW samples.

- Salicylic acid

Acetylsalicylic acid, also known as aspirin, is one of the most commonly used pharmaceuticals, due to its analgesic, antipyretic, anti-inflammatory and anticoagulant properties. Daily doses are 1.2-4 g for analgesic and antipyretic applications, and up to 8 g for anti-inflammatory treatment of arthritis (Zaugg et al., 2001). After oral administration, acetylsalicylic acid is hydrolyzed to SA by liver and blood esterases, and a 17.2% of the dose is excreted as SA in urine (Navarro et al., 2011).

- Atenolol

Atenolol is a cardioselective beta-blocking drug with antihypertensive properties. It's used to treat high blood pressure, to prevent angina and after heart attacks. It works by relaxing blood vessels to improve blood flow and decrease blood pressure. It has a relatively low bioavailability (50%) because of low absorption in the gastrointestinal tract. Due to its lack of biotransformation, 90% is excreted unchanged in humans (Tabacova and Kimmel, 2002).

- Cotinine and Hydroxycotinine (OH-COT)

Cotinine and OH-COT are alkaloid metabolites of nicotine, a stimulant drug found in the leaves of tobacco plants that acts as an insecticide. Nicotine is responsible for the addictive properties of tobacco smoking, its main route of administration, although it can also be absorbed orally and transdermal. Both of them are also excreted as glucoronides, howerverOH-COT glucuronide is very unstable in WW and transforming totally into OH-COT within 50 hours (Rodriguez-Alvarez et al.,

2014). Cotinine is responsible for 12.5% of the dose excreted in urine, and OH-COT and its glucuronide, for 44.5% (Hukkanen et al., 2005)

- 11-nor-9-carboxy-tetrahydrocannabinol (THC-COOH)

THC-COOH is a THC metabolite. THC is the primary hallucinogenic constituent of Cannabis, and is the responsible for its psychotropic effects. There are several main types of cannabis products, including herb (marijuana), resin (hashish) and oil (hashish oil). THC-COOH is its main metabolite excreted in urine, with an average of 0.6% of the amount of THC being excreted as THC-COOH (Castiglioni et al., 2011).

- Cocaethylene

CET is a cocaine metabolite, and it accounts for 0.7% of the dose excreted in urine. Cocaine is an alkaloid extracted from the leaves of Erythroxylon coca bush, and is one of the most abused illicit drugs. It can be used as cocaine hydrochloride, usually injected or snorted, or as free base (crack), which is smoked. The use of a metabolite instead of the parent compound excludes from consumption calculations the amounts of cocaine disposed directly in WW (Castiglioni et al., 2011). The internal standard used for signal correction was cocaethylene-d3.

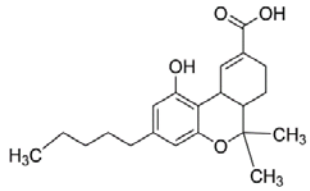
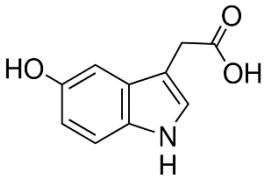
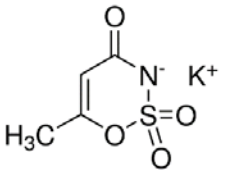
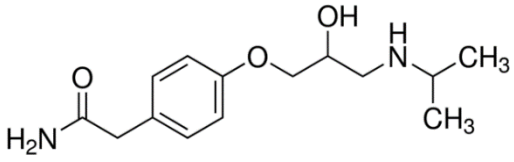
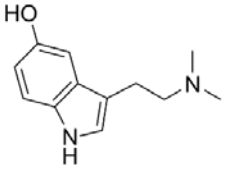
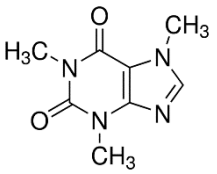
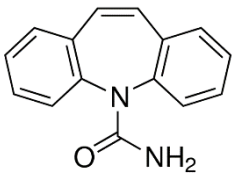
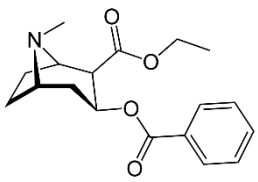
- Morphine

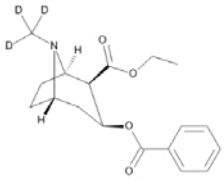
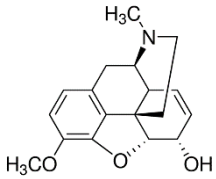
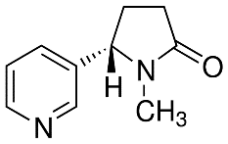
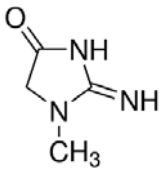
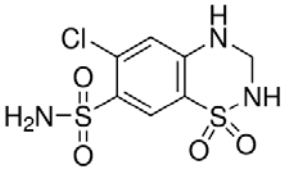
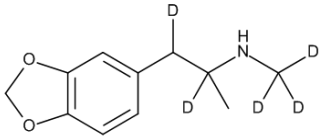
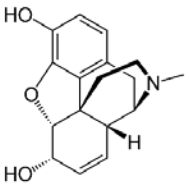
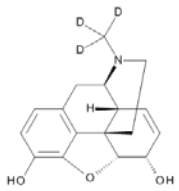
Morphine is a metabolic residue of heroin. Heroin is one of the most dangerous drugs of abuse, and is usually administrated as the hydrochloride salt by intravenous or subcutaneous injection, nasal insufflation or inhalation. 4.2% of the dose is recovered as free morphine in urine, and a 38.3% as conjugated morphine, which is hydrolyzed back to its free form by fecal bacteria (Castiglioni et al., 2011). The internal standard used was morphine-d3

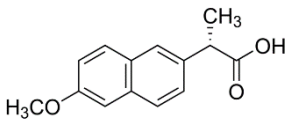
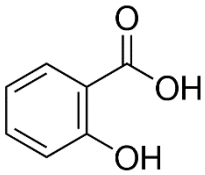
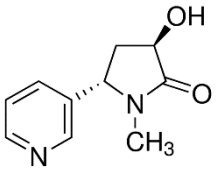
- Bufotenine

Bufotenine is a fast-acting and potent hallucinogen that can be found in the venom of some psychoactive toads and also in some plants, with an activity similar to Lysergic acid diethylamide (LSD) (Costa et al., 2005) Between 1 and 6% of the dose is recovered as bufotenine in urine (Shen et al., 2010). The internal standard used was MDMA-d5.

Table S7.1. Analytes studied, with their CAS number, formula and molecular weight.

	**11-nor-9-Carboxy-Δ9-THC** CAS Number: 104874-50-2 Empirical Formula: $C_{12}H_{28}O_4$ Molecular Weight: 344.44
	**5-Hydroxyindole-3-acetic acid** CAS Number: 54-16-0 Empirical Formula: $C_{10}H_9NO_3$ Molecular Weight: 191.18
	**Acesulfame K** CAS Number: 55589-65-3 Empirical Formula: $C_4H_4KNO_4S$ Molecular Weight: 201.24
	**Atenolol** CAS Number: 29122-68-7 Empirical Formula: $C_{14}H_{22}N_2O_3$ Molecular Weight: 266.34
	**Bufotenine** CAS Number: 487-93-4 Empirical Formula: $C_{12}H_{16}N_2O$ Molecular Weight: 204.27
	**Caffeine** CAS Number: 58-08-2 Empirical Formula: $C_8H_{10}N_4O_2$ Molecular Weight: 194.19
	**Carbamazepine** CAS Number: 298-46-4 Empirical Formula: $C_{15}H_{12}N_2O$ Molecular Weight: 236.27
	**Cocaethylene** CAS Number: 529-38-4 Empirical Formula: $C_{18}H_{23}NO_4$ Molecular Weight: 317.38

	**Cocaethylene-d3** CAS Number: 136765-30-5 Empirical Formula: $C_{18}H_{20}D_3NO_4$ Molecular Weight: 320.40
	**Codeine** CAS Number: 76-57-3 Empirical Formula: $C_{18}H_{21}NO_3$ Molecular Weight: 299.36
	**Cotinine** CAS Number: 486-56-6 Empirical Formula: $C_{10}H_{12}N_2O$ Molecular Weight: 176.22
	**Creatinine** CAS Number: 60-27-5 Empirical Formula: $C_4H_7N_3O$ Molecular Weight: 113.12
	**Hydrochlorothiazide** CAS Number: 58-93-5 Empirical Formula: $C_7H_8ClN_3O_4S_2$ Molecular Weight: 297.74
	**MDMA-D5** CAS Number: 136765-43-0 Empirical Formula: $C_{11}H_{10}D_5NO_2$ Molecular Weight: 198.27
	**Morphine** CAS Number: 57-27-2 Empirical Formula: $C_{17}H_{19}NO_3$ Molecular Weight: 285.34
	**Morphine-d3** CAS Number: 67293-88-3 Empirical Formula: $C_{17}H_{16}D_3NO_3$ Molecular Weight: 288.36

	**Naproxen** CAS Number: 22204-53-1 Empirical Formula: $C_{14}H_{14}O_3$ Molecular Weight: 230.26
	**Salicylic acid** CAS Number: 69-72-7 Empirical Formula: $C_7H_6O_3$ Molecular Weight: 138.12
	**trans-3'-Hydroxycotinine** CAS Number: 34834-67-8 Empirical Formula: $C_{10}H_{12}N_2O_2$ Molecular Weight: 192.21

Sample collection

Table S7.2. Description of the WWTP.

	PINEDO I	PINEDO II	QUART-BENÀGER
Population served	351,198	942,774	215,000
Municipalities	Valencia	Valencia + surrounding towns	Valencia + surrounding towns
Treated wastewaters	100 % Urban	100 % Urban	40% Urban/60 % Industrial
Treated technology			
Primary	Settlement Physicochemical	Settlement Physicochemical	Settlement Physicochemical
Secondary	Activated sludge	Nitrogen removal	Activated sludge/ P removal
Tertiary	Coagulation/flocculation Filtration	Coagulation/flocculation Filtration	Coagulation/flocculation
Influent characteristics			
Flow (m^3/day)	117,211	244,817	3,888
T (ºC)	17.2	17.3	18.4
pH	7.72	7.62	7.79
BOD_5 (mg/L)	223	264	367
COD (mg/L)	396	473	625
N (mg/L)	36.4	37.0	55.7
P (mg/L)	4.90	4.70	7.30
NH_4 (mg/L)	31.7	25.2	38.8

P: phosphorus; T: temperature; BOD_5: biochemical oxygen demand; COD: chemical oxygen demand; N: nitrogen; NH_4: ammonium.

LC-MS/MS Analysis

Chromatographic separation was performed using a Phenomenex Kinetex C18 column (1.7mm, 100 A, 50x2.11 mm) at 30ºC. Mobile phases were, for the positive mode A: water and B: MeOH with 0.1% formic acid, and for the negative mode A: water and B: MeOH with 10mM of ammonium formate, with gradients shown in **Table S7.3**.

Table S7.3. Mobile phase gradients.

POSITIVE		NEGATIVE	
Time (min)	**%B**	**Time (min)**	**%B**
0	30	0	30
5	95	0.5	30
12	95	12	95
		20	95

Mass spectrometry was performed by an Agilent 6410 triple quadrupole mass spectrometer with positive and negative mode electrospray ionization with the conditions shown in **Table S7.4**.

Table S7.4. LC-MS/MS conditions.

Volume	5 µL
Draw Position	3mm
LIQUID CHROMATOGRAPHY	
Flow	0.2 mL/min
Temperature	30ºC
SOURCE	
Gas T	300ºC
Gas Flow	10 L/min
Nebulizer	20 psi
Capillary Voltage	4000 V

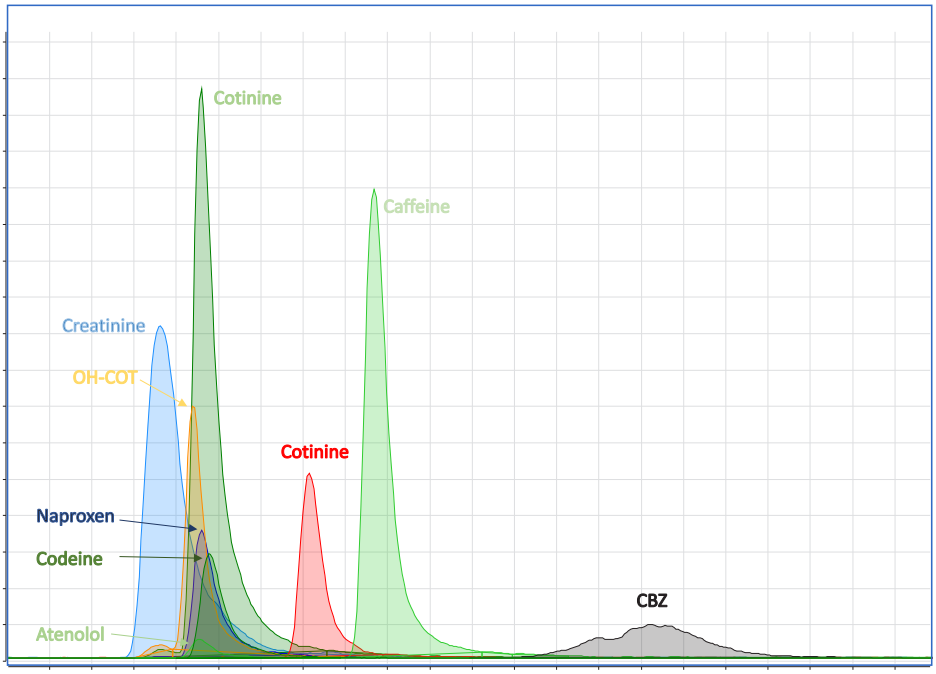
All samples and standards were analyzed using mass spectrometry with a multiple-reaction monitoring (MRM) mode of acquisition (Several transitions, one for quantification (in bold) and another ones for confirmation, were used for almost every analyte, as shown in SC, **Table S7.5**).

Table S7.5. Transitions, fragmentors, collision energies and polarity used in MS/MS.

Compound Name	Precursor Ion	Product Ion	Fragmentor	Collision Energy	Polarity
CET-d3	**321**	**199**	**132**	**17**	**Positive**
	321	85	132	25	Positive
CET	**318**	**196**	**132**	**17**	**Positive**
	318	82	132	29	Positive
Codeine	300	152	164	78	Positive
	300	**115**	**164**	**90**	**Positive**
Morphine-d3	289	165	162	45	Positive
	289	**152**	**162**	**73**	**Positive**
Morphine	286	165	152	45	Positive
	286	**152**	**152**	**69**	**Positive**
Atenolol	267	91	94	50	Positive
	267	**77**	**94**	**74**	**Positive**
CBZ	**237**	**194**	**104**	**18**	**Positive**
	237	192	104	22	Positive
Naproxen	**231**	**185**	**96**	**10**	**Positive**
	231	170	96	26	Positive
	231	115	96	70	Positive
Bufotenine	205	160	98	14	Positive
	205	**58**	**98**	**10**	**Positive**
MDMA-D5	**199**	**165**	**88**	**9**	**Positive**
Caffeine	**195**	**138**	**94**	**18**	**Positive**
	195	42	94	38	Positive
OH-COT	193	134	94	18	Positive
	193	**80**	**94**	**30**	**Positive**
	193	53	94	66	Positive
5-HIAA	**192**	**146**	**84**	**14**	**Positive**
	192	91	84	42	Positive
Cotinine	177	98	94	22	Positive
	177	**80**	**94**	**26**	**Positive**
Creatinine	**114**	**44**	**106**	**18**	**Positive**
	114	43	106	50	Positive
THC-COOH	**343**	**299**	**167**	**13**	**Negative**
	343	245	167	25	Negative
	343	191	167	20	Negative
HCTZ	**295**	**268**	**140**	**10**	**Negative**
	295	204	140	10	Negative
	295	77	140	26	Negative
Acesulfame	**161**	**82**	**84**	**10**	**Negative**
	161	77	84	34	Negative
SA	**137**	**93**	**86**	**10**	**Negative**

Transitions marked in bold are those used for quantification

(A)



(B)

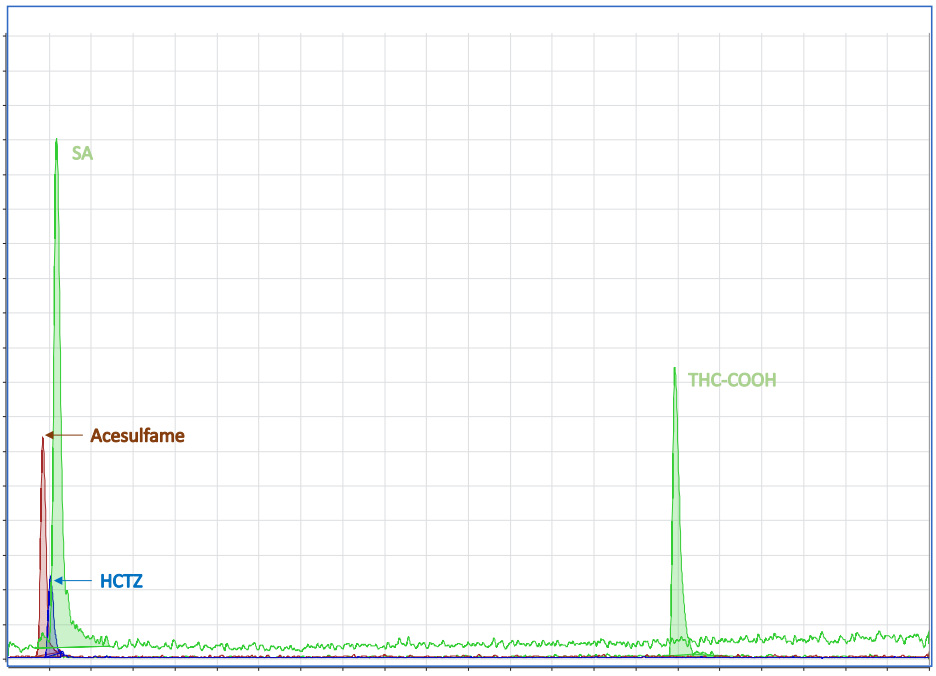


Fig. S7.2. UHPLC-MS/MS chromatograms corresponding to the calibration standard of 100 ng/L (A) positive ESI mode and (B) negative ESI mode.

Consumption, metabolisms and excretion data

Equations used for creatinine calculations:

- **Ix equation:**
  Creatinine (mg/day) = 879.89 + (12.51 x weight) - (6.19 x age) + 34.51 (if black) - 379.42 (if female)
- **Fotheringham equations:**
  Male/nonblack: Creatinine (mg/day) = 1307.3 + (23.1 x age) - (0.3 x age^2)
  Female/nonblack: Creatinine (mg/day) = 1051.3 + (5.3 x age) - (0.1 x age^2)

- **Walser equations:**
  Male: Creatinine (mg/day) = (28.2-0.172 x age) x weight
  Female: Creatinine (mg/day) = (21.9-0.115 x age) x weight

Table S7.6. Estimations of daily creatinine excretion.

		Creatinine Excretion (mg/day)	Average (mg /inh day)
Ix	Male	1598	1321.5
	Female	1045	
Fotheringham	Male	1752	1425
	Female	1098	
Walser	Male	1648	1372.5
	Female	1097	

Table S7.7. Range and average of daily loads found in WW samples.

	Quart-Benàger (g/day)	Pinedo I (g/day)	Pinedo II (g/day)
5-HIAA	158.1-293.6	596.2-1072.0	1592.0-2279.8
	224.8	**863.5**	**1985.4**
Acesulfame	204.8-1198.4	70.6-406.7	722.8-1858.9
	519.4	**245.1**	**1266**
Atenolol	359.6-2522.2	2210.4-4672.3	4734.8-8982.6
	1451.2	**2942**	**6301.7**
Bufotenine	0.439-1.611	1.020-3.092	6.029-21.600
	0.856	**1.888**	**14.123**
Caffeine	1047.8-3270.6	2450.3-3346.0	6762.6-8538.3
	2324.1	**2781.2**	**7511**
CBZ	0.268-0.746	0.449-1.231	0.607-2.597
	0.498	**0.892**	**1.403**
CET	0.595-1.347	1.118-2.718	3.090-8.103
	0.882	**1.874**	**5.438**
Codeine	7.83-21.65	22.11-41.82	84.01-122.01
	15.06	**29.92**	**99.02**
Cotinine	62.47-88.26	139.90-171.90	330.88-444.23
	76.47	**153.55**	**383.93**
Creatinine	11.67-104.73	60.58-182.01	292.89-501.15
	58.74	**127.23**	**393.49**
HCTZ	586.4-865.5	1326.1-2515.3	4292.6-7555.3
	734.1	**2034**	**6082.8**
Morphine	29.68-61.57	55.46-136.27	148.97-380.89
	46.27	**95.16**	**265.86**
Naproxen	69.9-127.8	194.2-233.3	646.8-775.5
	90.4	**218.0**	**673.0**
OH-COT	66.37-81.86	154.65-199.83	413.20-530.61
	73.93	**175.34**	**450.94**
SA	297.9-876.7	727.8-1919.9	2124.9-5866.6
	516.2	**1514.9**	**3503.6**
THC-COOH	5.85-10.15	15.20-27.07	45.88-72.28
	7.87	**20.11**	**56.56**

Values in bold are the averages

Table S7.8. Measures of hydrochemical parameters and population estimations based on them.

		BOD (mg/L)	COD (mg/L)	total N (mg/L)	total P (mg/L)	Inh based on BOD	Inh based on COD	Inh based on N	Inh based on P
QB	**Wednesday**	340	535	54.1	6.87	225420	166268	215210	160758
	Thursday	320	561	45.8	6.24	221680	182172	190368	152568
	Friday								
	Saturday								
	Sunday	440	690	75.4	9.37	156699	115187	161115	117775
	Monday	500	1000	80.7	11.2	196975	184664	190751	155726
	Tuesday	480	822	70.3	10.4	226784	182047	199286	173423
	Average	**416**	**721.6**	**65.26**	**8.816**	**205512**	**166068**	**191346**	**152050**
P I	**Wednesday**		435				278261		
	Thursday		469				266190		
	Friday		367				214512		
	Saturday		310				170306		
	Sunday		389				228428		
	Monday		528				322629		
	Tuesday	230	596	43.9	33.7	313716	381063	359273	1622338
	Average		**442**				**265913**		
P II	**Wednesday**		716				1366318		
	Thursday		775				1302321		
	Friday		854				1412363		
	Saturday		569				879678		
	Sunday		711				1004404		
	Monday		747				1173444		
	Tuesday	460	834	56.8	7.2	1593179	1353986	1180338	880120
	Average		**743.71429**				**1213216**		

QB: Quart-Benàger; PI: Pinedo I; PII: Pinedo II

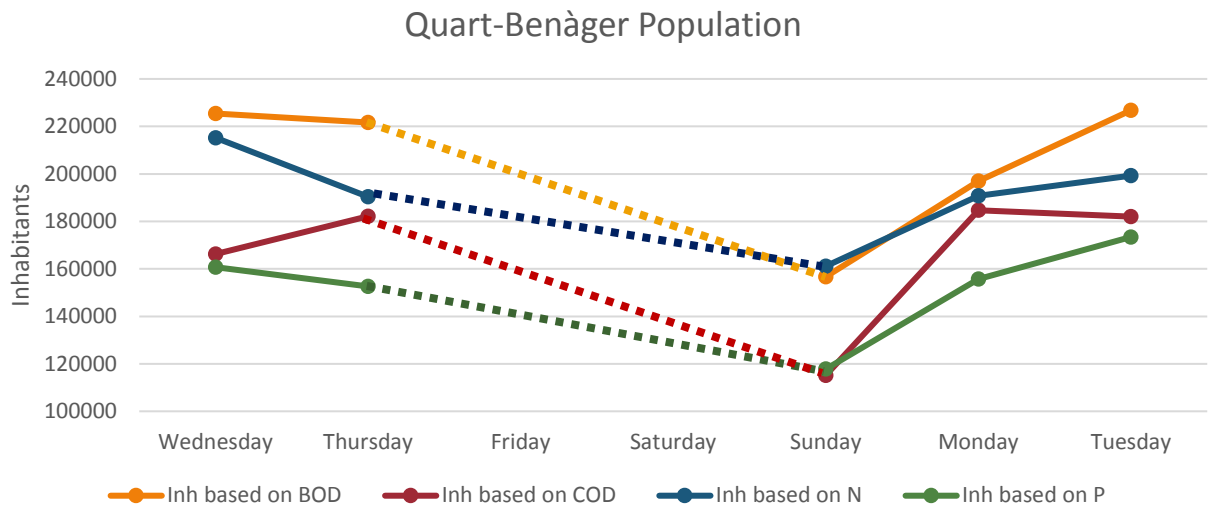


Fig. S7.3. Quart-Benàger Population based on hydrochemical parameters (doted lines are extrapolated).

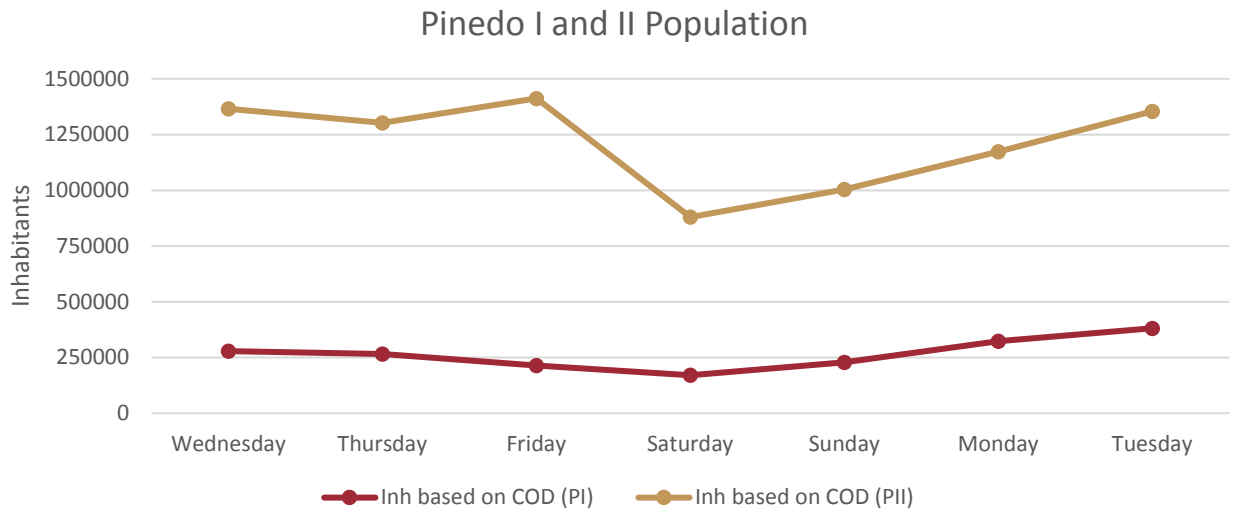


Fig. S7.4. Pinedo I and II Population based on COD.

Table S7.9. Number of inhabitants estimated with urine biomarkers

Quart-Benàger Inhabitants												
Method	**5-HIAA Dilute and shoot**	**SA SPE**	**Acesulfame SPE**	**Atenolol Dilute and shoot**	**Caffeine Dilute and shoot**	**CBZ SPE**	**Codeine Dilute and shoot**	**Cotinine Dilute and shoot**	**Creatinine Dilute and shoot**	**HCTZ SPE**	**Naproxen SPE**	**OH-COT Dilute and shoot**
Wednesday	63979	40535	20988	8837721	412957	56126	15587	164784	76	3367756	2747786	41949
Thursday	85342	66918	15513	5121841	383569	60682	18024	158272	74	3261340	3226138	43341
Friday	45965	45246	5072	7252004	299842	32406	10917	145839	38	2708358	1859796	36603
Saturday	49486	43071	5433	7558456	186214	31212	21430	182350	47	2281793	1824515	40073
Sunday	65481	26221	3587	3925078	132303	52774	15364	206035	23	2501907	2353143	40457
Monday	69042	22734	5015	1260028	294657	28429	7748	186747	8	2705622	1766359	37853
Tuesday	78051	31082	8066	1640699	344559	21804	15282	205497	31	3168266	2200143	45146
Average	**65335**	**39401**	**9096**	**5085118**	**293443**	**40490**	**14908**	**178503**	**43**	**2856434**	**2282554**	**40775**
Pinedo I Inhabitants												
Wednesday	239553	146534	5938	12697926	422474	97297	41383	372124	133	7527490	5783017	99946
Thursday	302255	140952	7123	7946004	348098	71519	21876	326578	107	5647476	4904624	88548
Friday	280960	133631	4987	7745226	353968	96682	25059	360214	111	5160073	5248628	96049
Saturday	177346	128430	4941	9973730	309375	48977	26748	334439	54	6539974	5356820	85290
Sunday	173318	55553	1236	16371776	313470	36523	25237	330064	77	8800700	5292558	87706
Monday	311623	64011	1633	8318259	325561	56484	33256	401278	44	9787255	6063312	110207
Tuesday	272137	140240	4196	9107368	385154	100060	33718	384440	123	11937683	5891259	109147
Average	**251028**	**115622**	**4293**	**10308613**	**351157**	**72506**	**29611**	**358448**	**93**	**7914379**	**5505745**	**96699**
Pinedo II Inhabitants												
Wednesday	630141	425807	31652	31474704	1078064	49326	116066	944766	365	16702779	19583512	292636
Thursday	662739	447771	32555	23352838	987368	57984	94901	845461	361	21443938	16501655	258298
Friday	607459	241185	21068	24328885	926398	55166	120738	863861	290	21098845	15877007	244801
Saturday	529715	257018	27159	23242013	941149	103521	95880	944354	213	26888626	17360002	236638
Sunday	462794	174131	13324	17948818	853859	165260	91676	1037023	225	24878302	16332100	231278
Monday	574881	163783	12659	17629445	967490	211157	83139	865903	235	25276255	16437112	249343
Tuesday	572280	162181	16787	16590636	884196	156158	83568	772413	316	29390136	16881893	227881
Average	**577144**	**267411**	**22172**	**22081048**	**948361**	**114082**	**97995**	**896254**	**287**	**23668411**	**16996183**	**248696**

Table S7.10. ANOVA test made for the 3 WWTP. A p-value higher than 0.05 (in bold) indicates that there is no statistical difference between the weekly averages of the population estimations.

ANOVA Quart-Benàger Weekly Average															
P-Values	**SA**	**Acesulfame**	**Atenolol**	**Caffeine**	**CBZ**	**Codeine**	**Cotinine**	**Creatinine**	**HCTZ**	**Naproxen**	**OH-COT**	**BOD**	**COD**	**Total N**	**Total P**
5-HIAA	0.0009	0.0000	0.0008	0.0001	0.0088	0.0000	0.0000	0.0000	0.0000	0.0000	0.0007	0.0000	0.0000	0.0000	0.0000
SA		0.0003	0.0007	0.0000	0.0180	0.0013	0.0000	0.0000	0.0000	0.0000	**0.8147**	0.0000	0.0000	0.0000	0.0000
Acesulfame			0.0007	0.0000	0.0004	**0.0775**	0.0000	0.0034	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Atenolol				0.0011	0.0007	0.0007	0.0009	0.0007	**0.0724**	0.0300	0.0007	0.0009	0.0009	0.0009	0.0009
Caffeine					0.0000	0.0000	0.0132	0.0000	0.0000	0.0000	0.0000	0.0380	0.0061	0.0186	0.0029
CBZ						0.0013	0.0000	0.0000	0.0000	0.0000	**0.9629**	0.0000	0.0000	0.0000	0.0000
Codeine							0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Cotinine								0.0000	0.0000	0.0000	0.0000	**0.1282**	**0.2133**	**0.4647**	0.0176
Creatinine									0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HCTZ										0.0461	0.0000	0.0000	0.0000	0.0000	0.0000
Naproxen											0.0000	0.0000	0.0000	0.0000	0.0000
OH-COT												0.0000	0.0000	0.0000	0.0000
BOD													0.0169	**0.2896**	0.0011
COD														**0.0532**	**0.2804**
Total N															0.0018

ANOVA Pinedo I Weekly Average												
P-Values	**SA**	**Acesulfame**	**Atenolol**	**Caffeine**	**CBZ**	**Codeine**	**Cotinine**	**Creatinine**	**HCTZ**	**Naproxen**	**OH-COT**	**COD**
5-HIAA	0.0002	0.0000	0.0000	0.0026	0.0000	0.0000	0.0008	0.0000	0.0000	0.0000	0.0000	**0.6709**
SA		0.0000	0.0000	0.0000	0.0307	0.0001	0.0000	0.0000	0.0000	0.0000	**0.2343**	0.0003
Acesulfame			0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000
Atenolol				0.0000	0.0000	0.0000	0.0000	0.0000	**0.1381**	0.0018	0.0000	0.0000
Caffeine					0.0000	0.0000	**0.7080**	0.0000	0.0000	0.0000	0.0000	0.0171
CBZ						0.0012	0.0000	0.0000	0.0000	0.0000	0.0410	0.0000
Codeine							0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Cotinine								0.0000	0.0000	0.0000	0.0000	0.0075

Creatinine								0.0000	0.0000	0.0000	0.0000	
HCTZ									0.0236	0.0000	0.0000	
Naproxen										0.0000	0.0000	
OH-COT											0.0000	

ANOVA Pinedo II Weekly Average

P-Values	**SA**	**Acesulfame**	**Atenolol**	**Caffeine**	**CBZ**	**Codeine**	**Cotinine**	**Creatinine**	**HCTZ**	**Naproxen**	**OH-COT**	**COD**
5-HIAA	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SA		0.0002	0.0000	0.0000	0.0122	0.0033	0.0000	0.0001	0.0000	0.0000	**0.6960**	0.0000
Acesulfame			0.0000	0.0000	0.0027	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Atenolol				0.0000	0.0000	0.0000	0.0000	0.0000	**0.5434**	0.0274	0.0000	0.0000
Caffeine					0.0000	0.0000	**0.2460**	0.0000	0.0000	0.0000	0.0000	0.0070
CBZ						**0.5306**	0.0000	0.0005	0.0000	0.0000	0.0002	0.0000
Codeine							0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Cotinine								0.0000	0.0000	0.0000	0.0000	0.0025
Creatinine									0.0000	0.0000	0.0000	0.0000
HCTZ										0.0017	0.0000	0.0000
Naproxen											0.0000	0.0000
OH-COT												0.0000

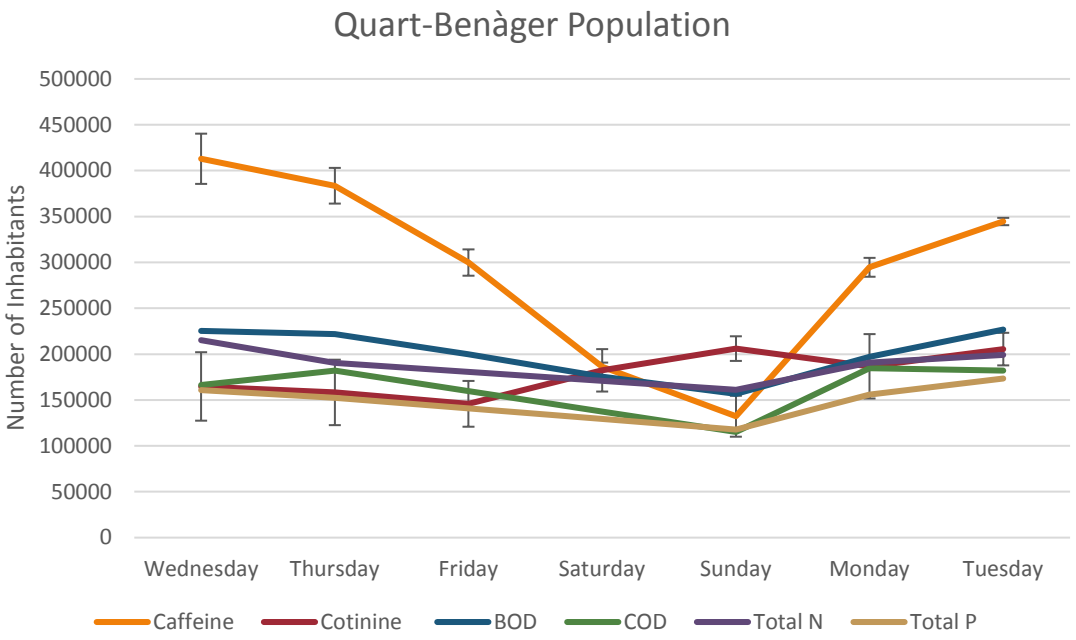


Fig. S7.5. Quart-Benàger Population based on urine biomarkers and hydrochemical parameters.

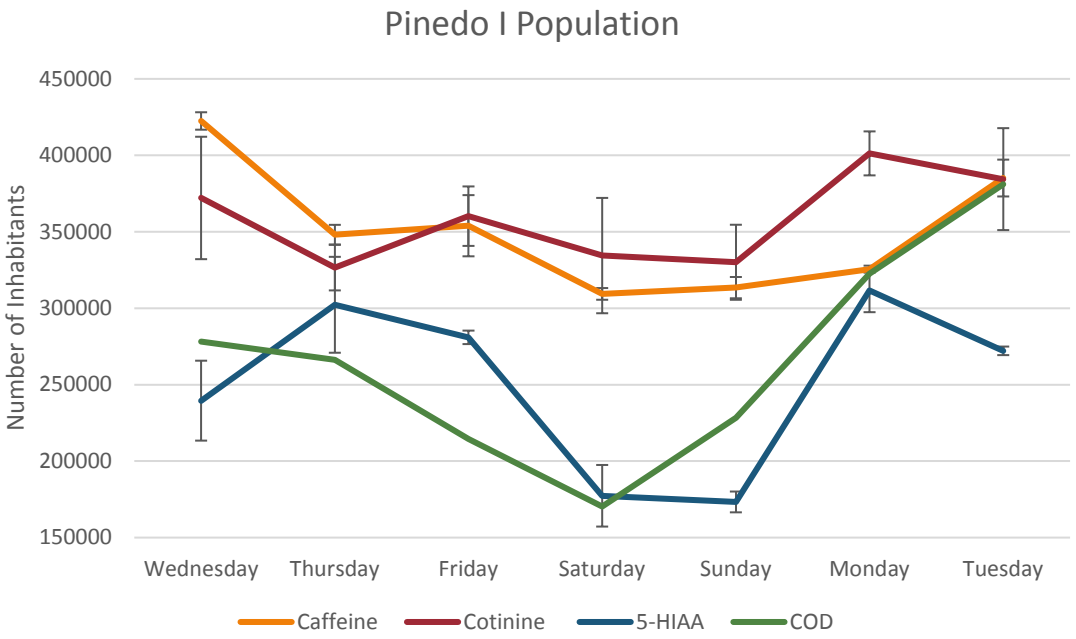


Fig. S7.6. Pinedo I Population based on urine biomarkers and hydrochemical parameters.

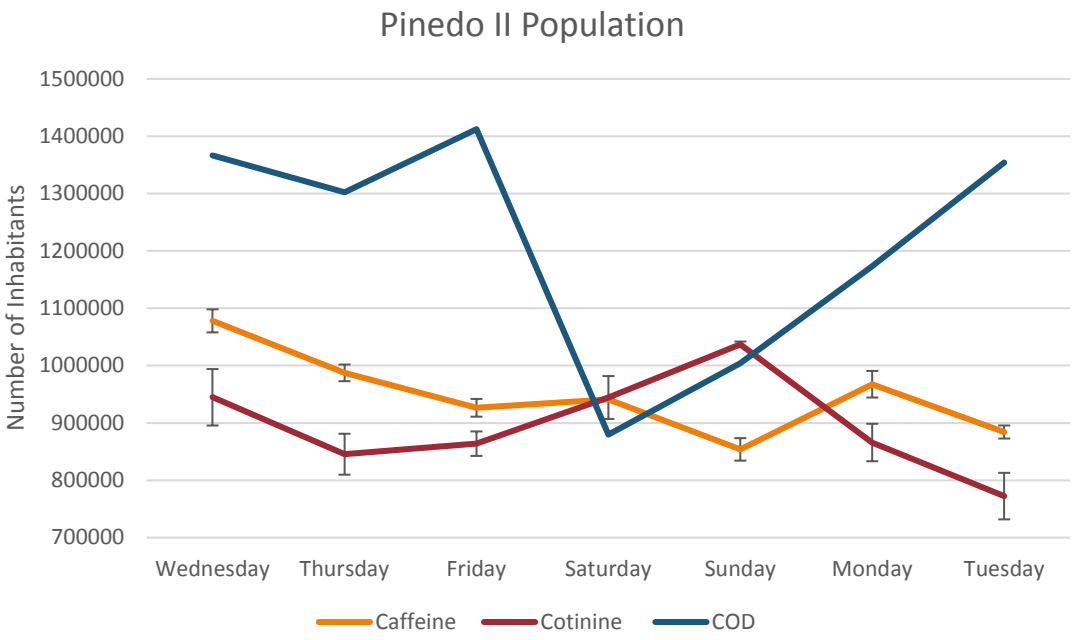


Fig. S7.7. Pinedo II Population based on urine biomarkers and hydrochemical parameters.

Table S7.11. ANOVA test for the daily averages of the population estimations.

ANOVA Quart-Benàger daily population						
P-values		**Cotinine**	**BOD**	**COD**	**Total N**	**Total P**
Wednesday	Caffeine	0.0270	0.0105	0.0061	0.0094	0.0058
	Cotinine		**0.4248**	**0.4608**	**0.5737**	**0.3922**
Thursday	Caffeine	0.0267	0.0071	0.0046	0.0050	0.0035
	Cotinine		**0.3617**	**0.8913**	**0.9149**	**0.3694**
Friday	Caffeine	0.0313	0.0102	0.0052	0.0072	0.0041
	Cotinine		**0.3120**	**0.6157**	**0.7223**	**0.2736**
Saturday	Caffeine	**0.2767**	**0.0778**	0.0043	0.0413	0.0032
	Cotinine		**0.1750**	**0.0565**	**0.1484**	0.0470
Sunday	Caffeine	0.0291	**0.2620**	**0.3913**	**0.2093**	**0.4543**
	Cotinine		0.0164	0.0076	0.0181	0.0079
Monday	Caffeine	**0.1329**	0.0055	0.0043	0.0049	0.0027
	Cotinine		**0.5080**	**0.3469**	**0.4181**	**0.1579**
Tuesday	Caffeine	0.0204	0.0000	0.0000	0.0000	0.0000
	Cotinine		**0.3998**	**0.0551**	**0.1001**	0.0430

ANOVA Pinedo I daily population				
P-Values		**Cotinine**	**5-HIAA**	**COD**
Wednesday	Caffeine	**0.8178**	0.0105	0.0008
	Cotinine		**0.0944**	**0.1266**
	5-HIAA			**0.1712**
Thursday	Caffeine	**0.1065**	**0.1802**	0.0031
	Cotinine		**0.0822**	0.0107
	5-HIAA			**0.2454**
Friday	Caffeine	**0.0807**	0.0372	0.0101
	Cotinine		0.0129	0.0059
	5-HIAA			0.0022
Saturday	Caffeine	**0.1142**	0.0118	0.0004

	Cotinine		0.0244	0.0191
	5-HIAA			**0.6701**
Sunday	Caffeine	**0.1679**	0.0024	0.0033
	Cotinine		0.0264	0.0453
	5-HIAA			0.0075
Monday	Caffeine	0.0024	0.0138	0.0001
	Cotinine		0.0092	0.0063
	5-HIAA			**0.3876**
Tuesday	Caffeine	**0.1427**	0.0056	**0.6784**
	Cotinine		0.0227	**0.1208**
	5-HIAA			0.0000

ANOVA Pinedo II daily population			
P-Values		**Cotinine**	**COD**
Wednesday	Caffeine	**0.4858**	0.0024
	Cotinine		0.0256
Thursday	Caffeine	**0.5981**	0.0011
	Cotinine		0.0018
Friday	Caffeine	0.0470	0.0005
	Cotinine		0.0020
Saturday	Caffeine	**0.3553**	0.0006
	Cotinine		**0.2475**
Sunday	Caffeine	0.0015	0.0084
	Cotinine		0.0004
Monday	Caffeine	**0.1082**	0.0063
	Cotinine		0.0048
Tuesday	Caffeine	**0.1162**	0.0003
	Cotinine		0.0002

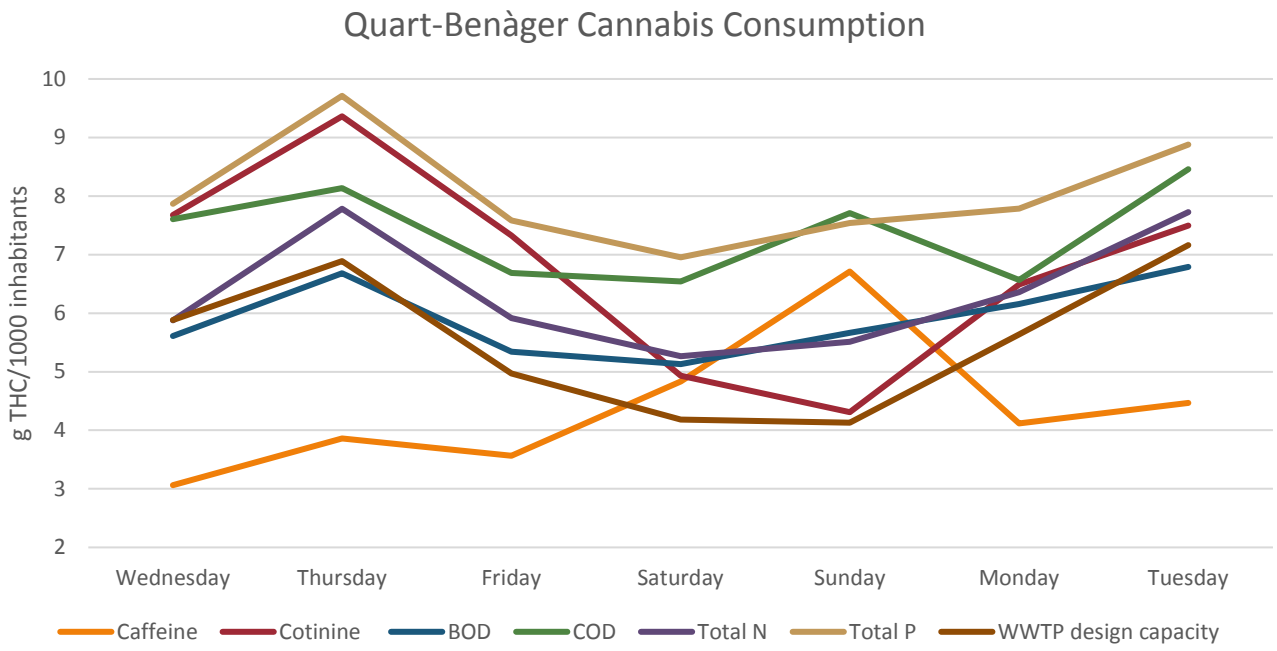


Fig. S7.8. Daily cannabis consumption in Quart-Benàger using different population estimations.

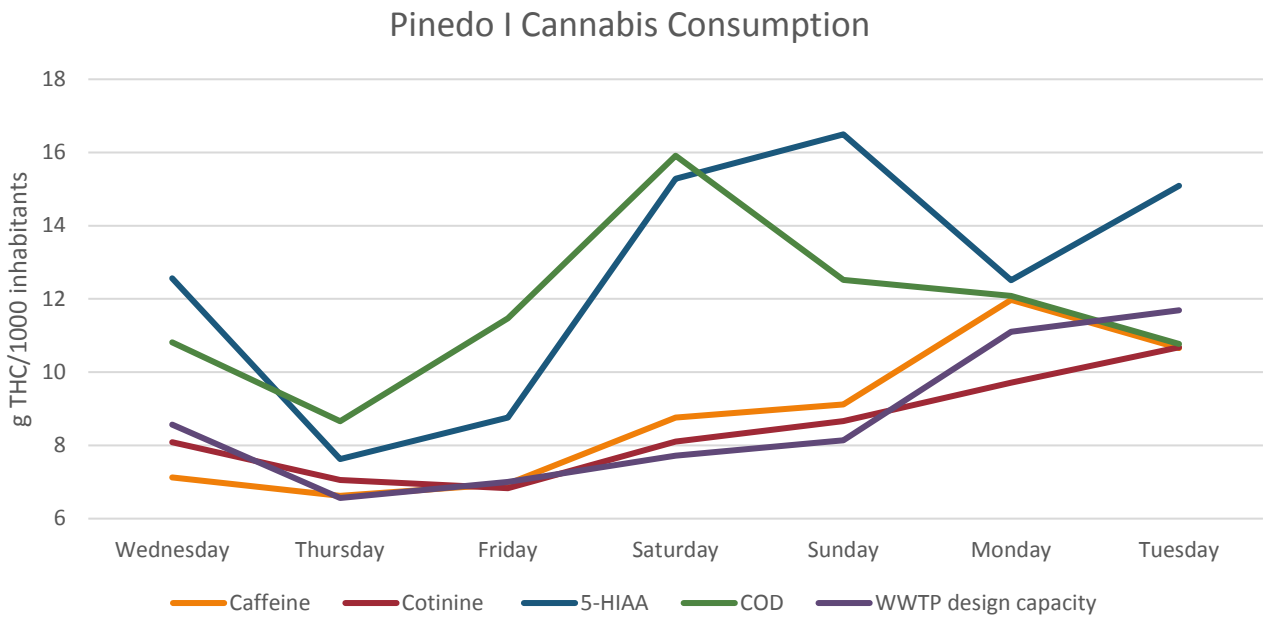


Fig. S7.9. Daily cannabis consumption in Pinedo I using different population estimations.

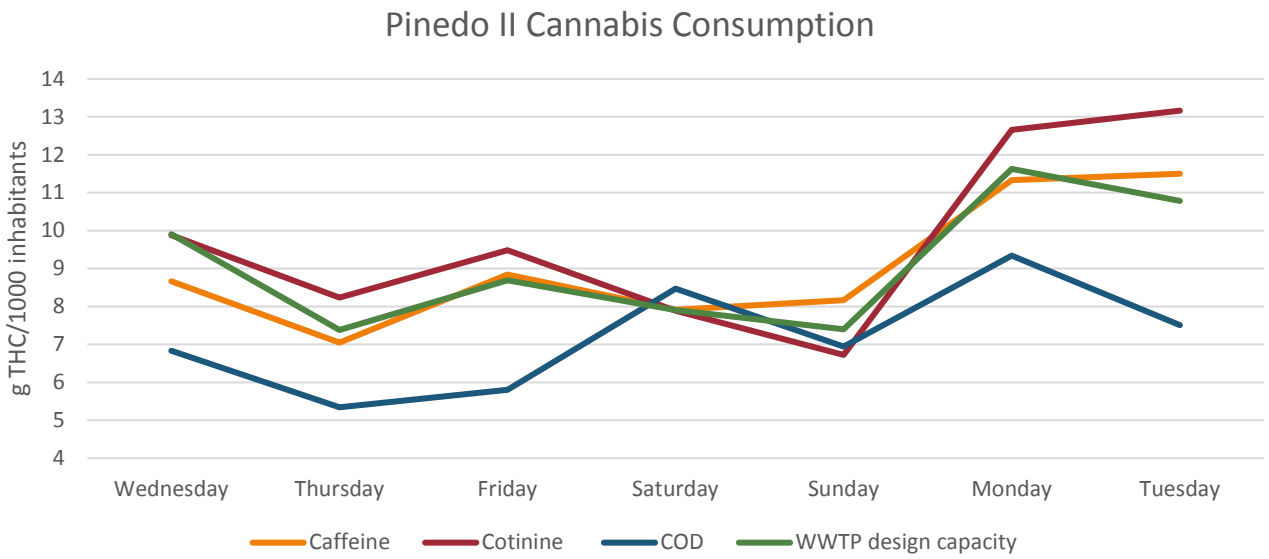


Fig. S7.10. Daily cannabis consumption in Pinedo II using different population estimations.

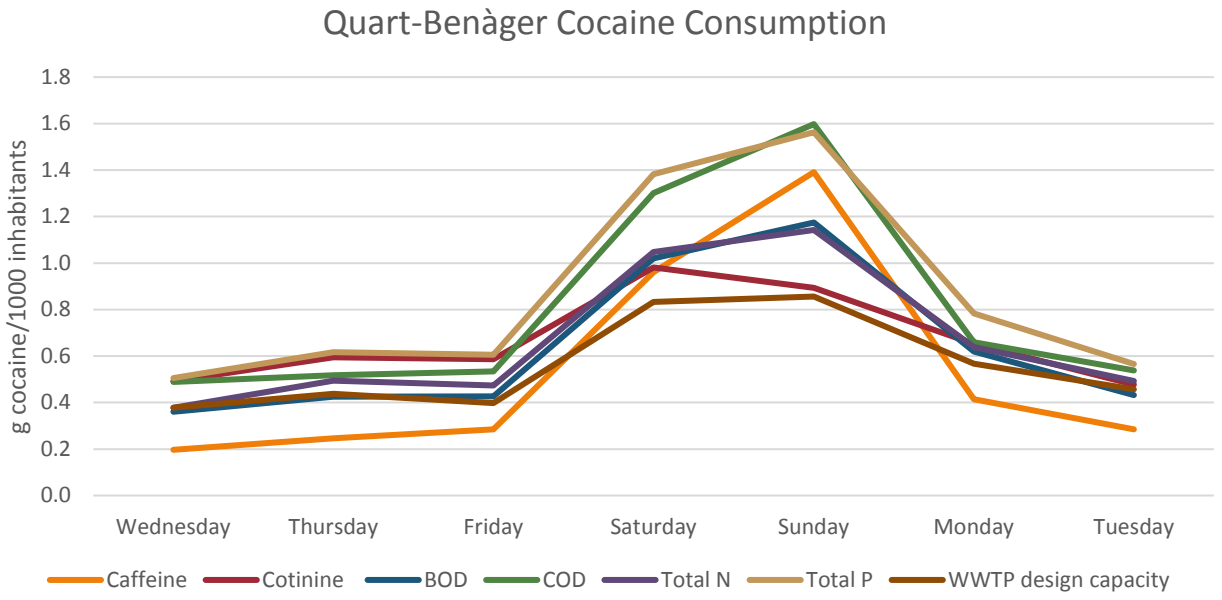


Fig. S7.11. Daily cocaine consumption in Quart-Benàger using different population estimations.

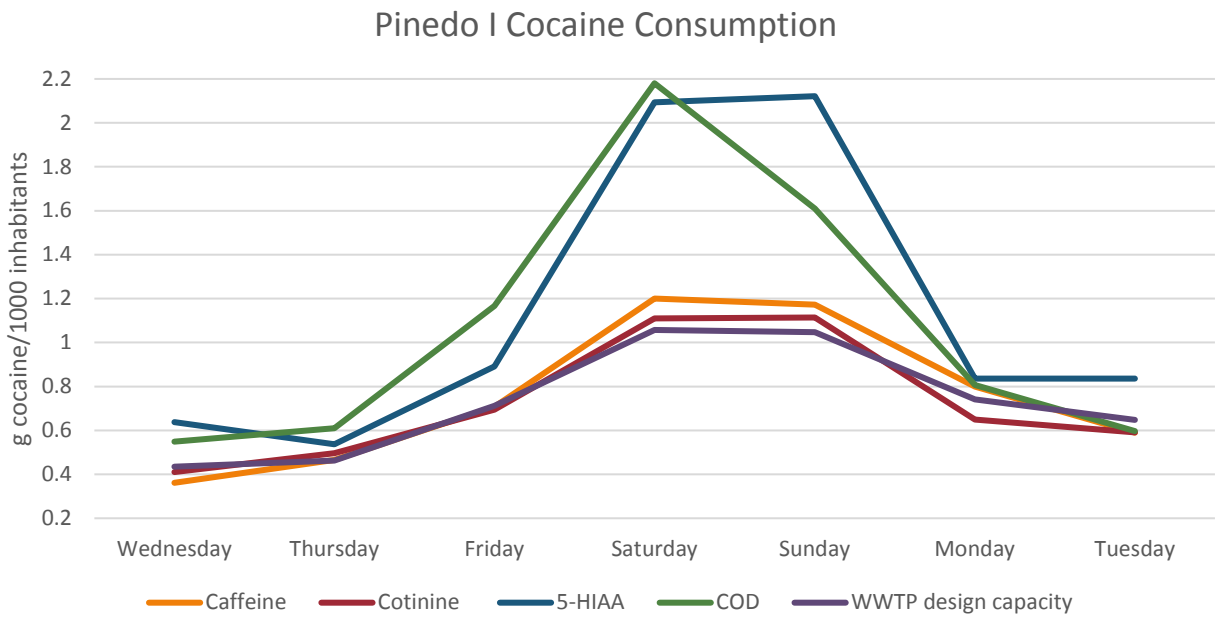


Fig. S7.12. Daily cocaine consumption in Pinedo I using different population estimations.

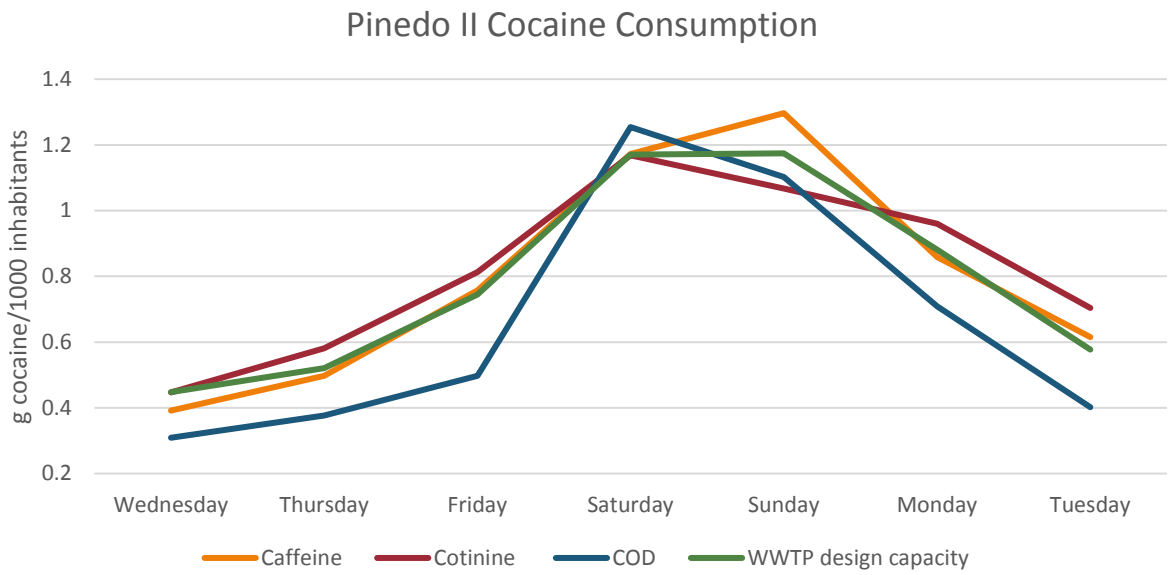


Fig. S7.13. Daily cocaine consumption in Pinedo II using different population estimations.

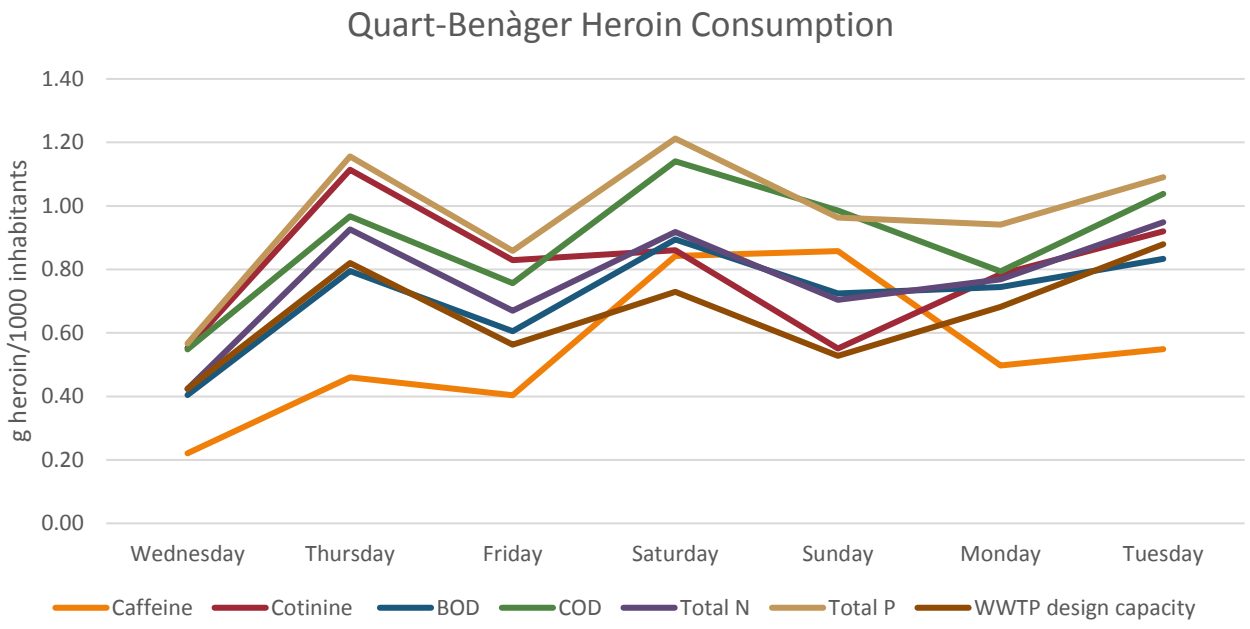


Fig. S7.14. Daily heroin consumption in Quart-Benàger using different population estimations.

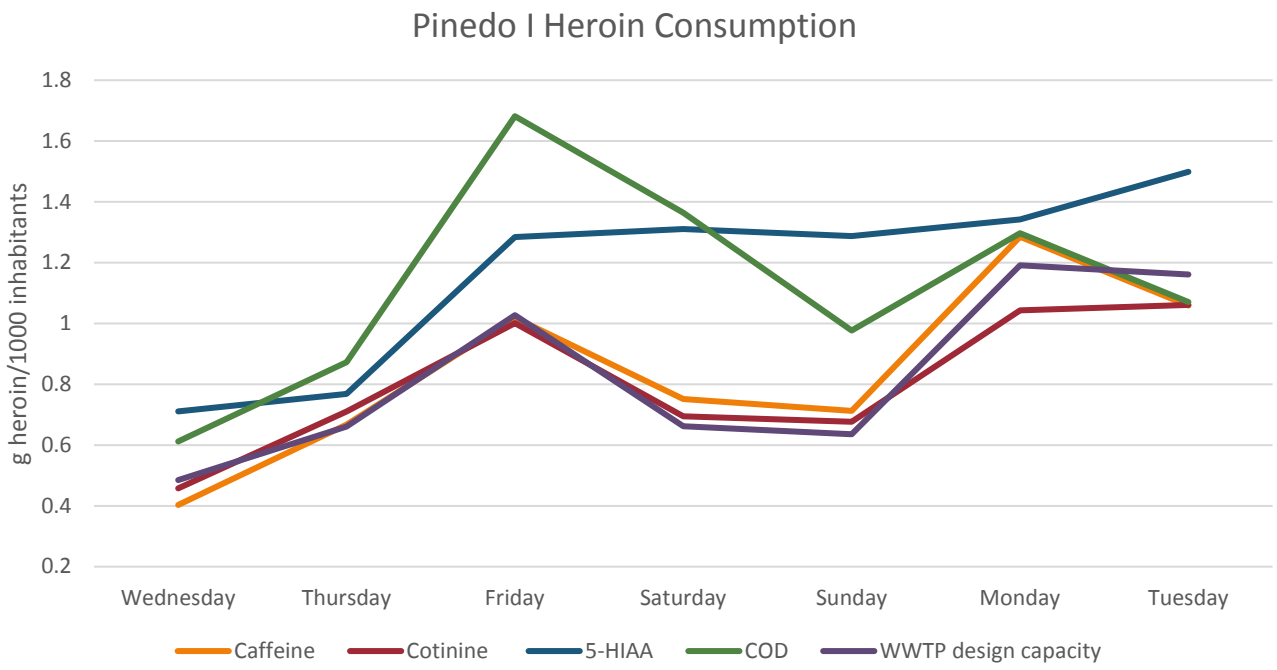


Fig. S7.15. Daily heroin consumption in Pinedo I using different population estimations.

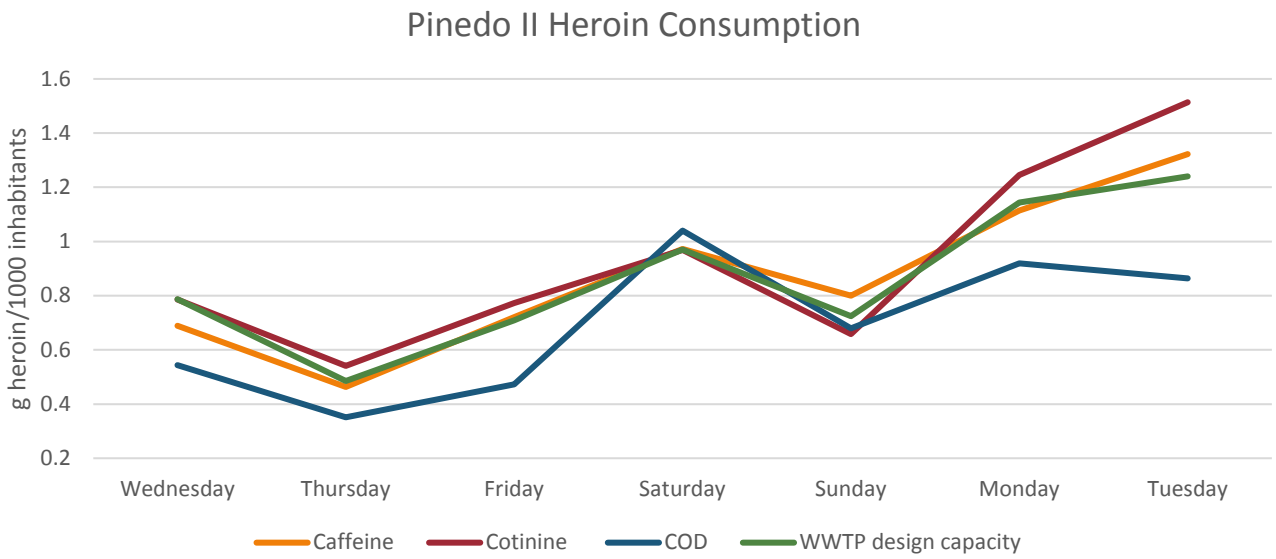


Fig. S7.16. Daily heroin consumption in Pinedo II using different population estimations.

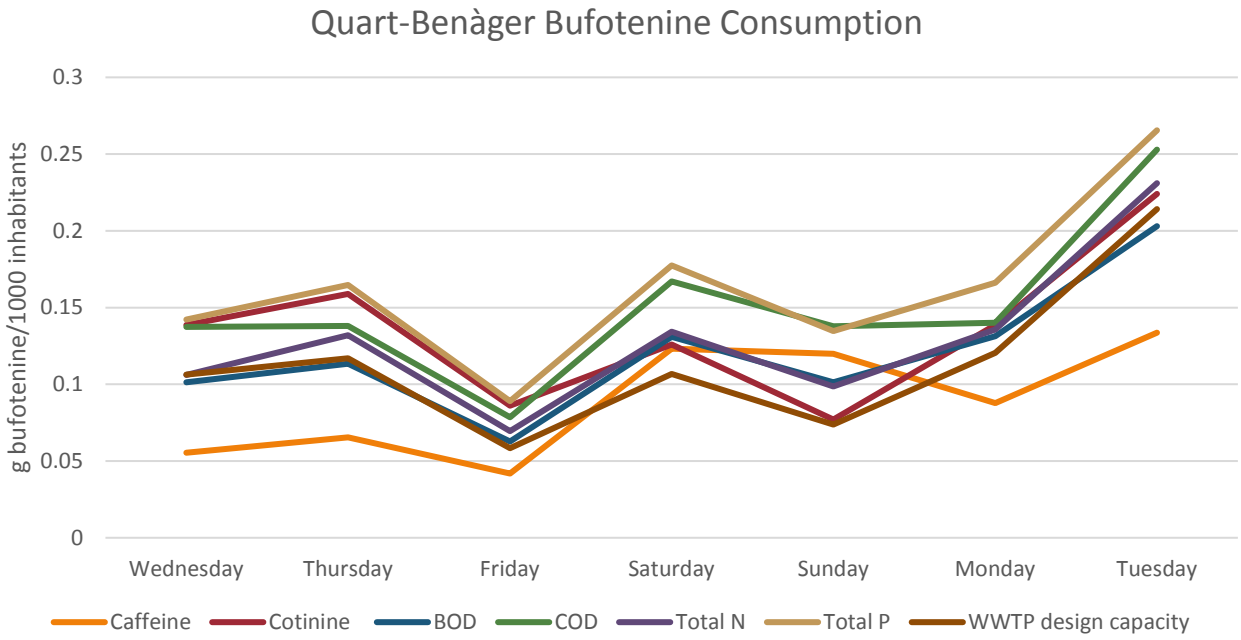


Fig. S7.17. Daily bufotenine consumption in Quart-Benàger with different population estimations.

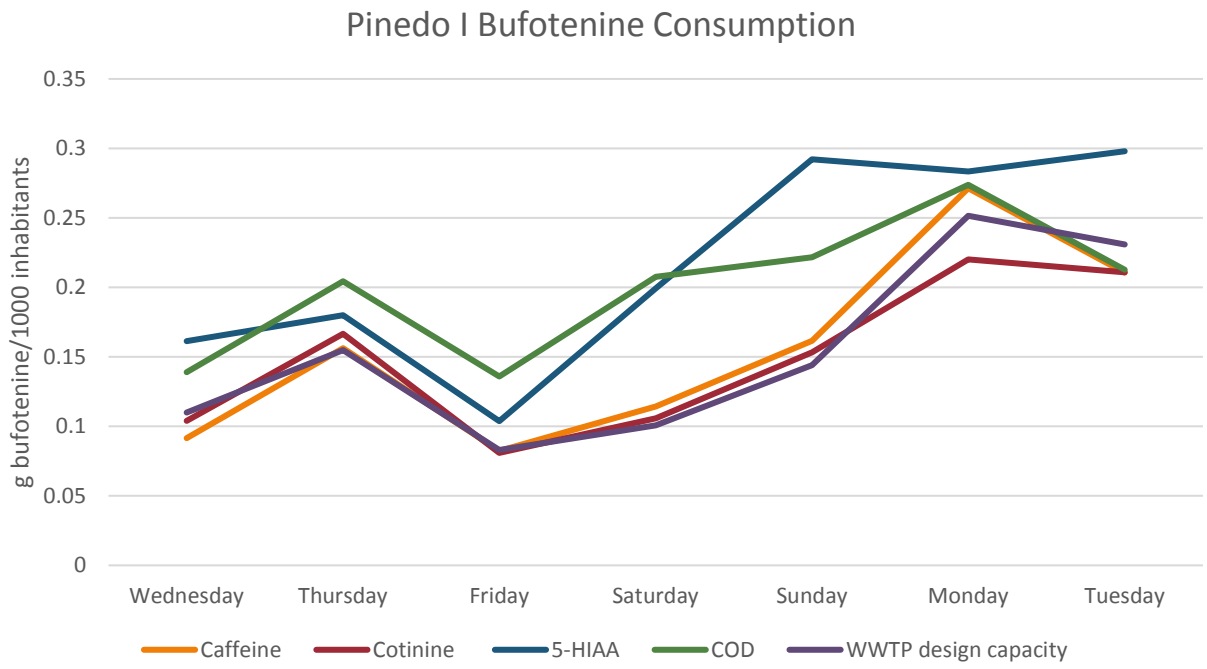


Fig. S7.18. Daily bufotenine consumption in Pinedo I with different population estimations.

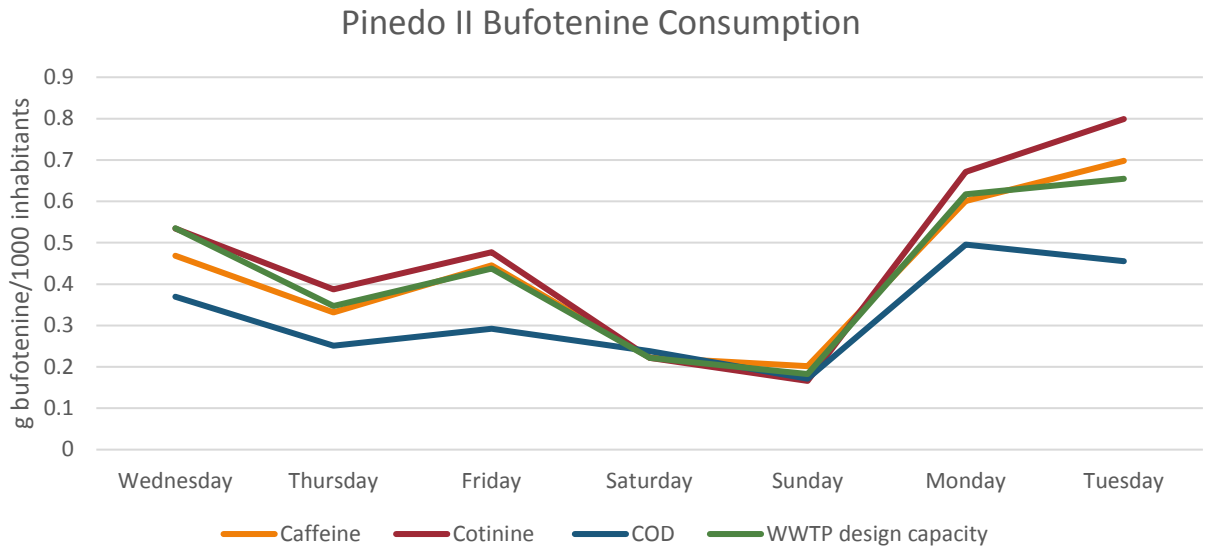


Fig. S7.19. Daily bufotenine consumption in Pinedo II with different population estimations.

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Capítulo 4

CAPÍTULO 4

FORENSÍA AMBIENTAL Y ALIMENTARIA

En el Capítulo 4 se abordan los problemas medioambientales suscitados por estos compuestos, mediante un estudio integrado de la contaminación generada. En este apartado se analizan numerosos aspectos producidos a causa de esta contaminación. La relación entre la presencia de estos compuestos y las características de la población se establece mediante el uso de un Sistema de Información Geográfica (SIG). La relación entre los niveles de estos compuestos y los parámetros de calidad de estas aguas superficiales se determina mediante las correlaciones de Pearson. En este capítulo se presenta el trabajo llevado a cabo en la Universidad de Bath, en Inglaterra, durante un periodo en el cual se estimó la bio y fotodegradación y la transformación enantioselectiva de un antidepresivo como es la fluoxetina en aguas superficiales del río Avon y lodos activos de la WWTP correspondiente a esa área. En este estudio también se evaluó la toxicidad de la fluoxetina a diferentes niveles tróficos mediante estudios in vivo. En la parte final del capítulo se evalúa el posible impacto en el ser humano debido al consumo de distintos productos alimenticios que pudieran contener cannabis o sus metabolitos. Este último problema, derivado de la alimentación del ganado con derivados del cáñamo, es un tema candente que ha preocupado a la *European Food Safety Authority* (EFSA), hasta el punto de destacar en un comunicado, la necesidad de estos estudios (EFSA 2011). El capítulo se organiza en tres publicaciones que cubren esta temática.

- Publicación científica 8. *Assessing drugs of abuse distribution in Turia River based on geographic information system and liquid chromatography mass spectrometry*
- Publicación científica 9. *Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance*
- Publicación científica 10. *Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety*

PUBLICACIÓN CIENTÍFICA 8

Assessing drugs of abuse distribution in Turia River based on geographic information system and liquid chromatography mass spectrometry

M.J. Andrés-Costa, J. Pascual-Aguilar, V. Andreu, Y. Picó

Environmental Pollution (en fase de revisión)

ASSESSING DRUGS OF ABUSE DISTRIBUTION IN TURIA RIVER BASED ON GEOGRAPHIC INFORMATION SYSTEM AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

María Jesús Andrés-Costa[(1)]*, Juan Pascual-Aguilar[(2)], Vicente Andreu[(2)] and Yolanda Picó[(1)]

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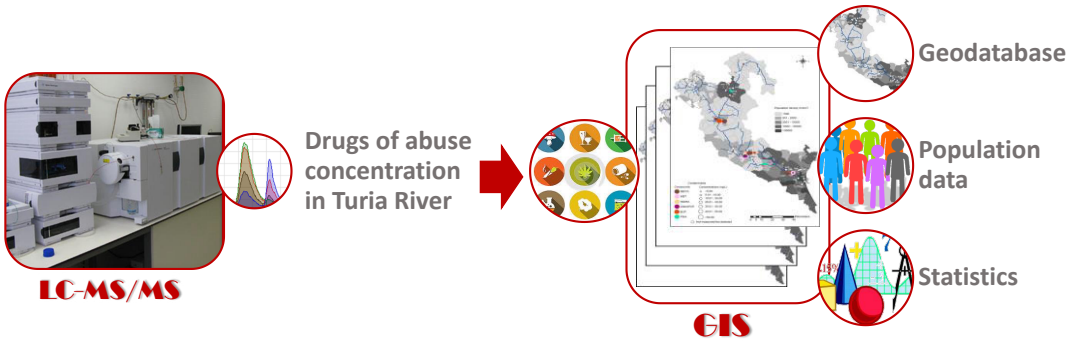
Highlights

- BECG, MET and MDMA frequent at ng/L in the Turia River (Spain).
- First detection of PMA, BUF and 4-MeO-PCP in surface waters.
- Descriptive GIS assessment related the occurrence of these drugs to high population densities.
- No acute environmental risk might be expected in aquatic organisms.

Graphical abstract



Abstract

Drugs of abuse are continuously discharged into wastewaters as part of their elimination process. Pollution at very low concentrations appears to be broad in environmental compartments near populated areas. A total of 42 drugs of abuse and metabolites were analysed in surface water samples collected in 2012 and 2013. Analysis was performed by solid phase extraction and liquid chromatography coupled mass spectrometry (SPE-LC-MS/MS). Analytical results of target compounds were georeferenced and integrated into a geographical information systems (GIS). Ecotoxicological risk of drugs of abuse detected in the Turia River was evaluated in this study by calculating risk quotient (RQ). In 2012, 6 compounds were detected in a total of 22 points. In 2013, 7 compounds were found in a total of 31 sampling sites, 4 of them also detected in 2012. The most frequent compound was benzoylecgonine (BECG), detected in 9 sampling points in 2012 and 8 in 2013, at an average concentration of 25.45 ng/L and 14.02 ng/L. Codeine (COD) reached maximum concentration of detected drugs of abuse (101.02 ng/L) in 2013. GIS provided the spatial incidence of drugs of abuse along the Turia River Basin. The distribution of these compounds in 2012 and 2013 shows that the highest concentrations and frequency of drugs of abuse run into places with highest population density. The RQ obtained from measured concentrations of detected drugs predict that no short-term environmental risk might be expected.

Capsule: The application of GIS shows, in a qualitatively way, that the presence of drugs of abuse is linked to large populations with more than 10000 inhabitants.

Keywords: mass spectrometry, Turia River, drugs of abuse, GIS

1. Introduction

There is a growing intererst in keeping intact natural resources to ensure their quality and sustainability (Petrie *et al.*, 2015; Rosi-Marshall *et al.*, 2015). Legislative innitiatives have been developed at regional, national and EU levels with the objective of preserving, protecting, and improving the quality and sustainable use of natural waters (Directive, 2000/60/EC). The most important pressure on these resources is the human development in its different faces (agriculture, industry, urbanization, infrastructures, etc.) to the point that some researchers has coined the "anthropocene" as a new geo-stratigraphic era (Dudgeon, 2011). River basins are one of the areas that better reflects these pressures and their effects (Belenguer *et al.*, 2014; Mastroianni *et al.*, 2016; Montuori *et al.*, 2015).

Emerging contaminants have been described as chemicals that had not previously been detected (or were previously found in far lesser concentrations) and include diversity substances with both industrial and domestic applications, among them, the most well known are drugs of abuse, pharmaceuticals or personal care products —all of them representatives of the anthropic impact. Drugs of abuse are widely consumed by the population and then, are continuously discharged into wastewaters unchanged or as metabolites. In fact, wastewater-based epidemiology has become an alternative tool to assess their use in communities (in a direct, quick and objective way) that complements and offers advantages over traditional surveys (Andrés-Costa *et al.*, 2014; Gatidou *et al.*, 2016; Ort *et al.*, 2014; Rodríguez-Álvarez *et al.*, 2015).

The presence of drugs of abuse in the environment raises increasing concern due to its already well-demonstrated adverse effects in population (Raktim Pal *et al.*, 2013). Insufficiently treated municipal wastewater discharges are the main route for surface

water contamination. Pollution by drugs of abuse at very low concentrations appears to be broad in environmental compartments near populated areas, and has been demonstred by various national and international field studies (Berset *et al.*, 2010; Jiang *et al.*, 2015; Mendoza, Rodríguez-Gil*, et al.*, 2014; Robles-Molina *et al.*, 2014; Rodayan *et al.*, 2015; Senta *et al.*, 2015; Valcárcel *et al.*, 2012) becoming a global factor all over the world. Although reported concentrations are negligible for human beings, situation could bring potential risks for aquatic biota threaten the stability and diversity of the ecosystem services (Catalá *et al.*, 2015; van der Aa *et al.*, 2013). There are already some studies carried out in Valencian Community that pointed out the ubiquitous presence of these compounds in all the areas studied. Vazquez-Roig *et al.* (2010, 2011) studied the presence and distribution of 15 widely used drugs of abuse in soil, water and sediment in l'Albufera and Oliva-Pego wetlands. Cocaine (COC) and metabolites as benzoylecgonine (BECG) and ecgonine methyl ester (ECME), amphetamines-type stimulants as amphetamine (AMP) and ecstasy (MDMA), methadone (METH), codeine (COD), morphine (MOR), and 11-Nor-9-carboxy-Δ_9-tetrahidrocannabinol (THC-COOH) were detected in water. Osorio *et al.* (2016) carried out a study to determine the concentration and risk of pharmaceuticals in freshwater systems related to the pouplation density in Iberian Rivers. Mastroianni *et al.*(2016) investigate the occurrence of 22 drugs of abuse and their metabolites in four Spanish river basins. The estimation of ecotoxicological hazards using risk quotient (RQ) ratios and a concentration addition model showed that these compounds posse certain risk to aquatic organisms. COC, ephedrine (EPH), MDMA, and METH were the most ubiquitous compounds, being present in more than 50% of the samples, but the results obtained did not show a clear relationship between the concentrations and characteristics of the river basin or riverside population.

Mapping techniques have provided a powerful tool for integrating information on the occurrence of these emerging contaminants that are related to landscape characteristics, infrastructure location and population individualities. Up to day, only few approaches to mapping emerging contaminants (mostly pharmaceutical) and their attenuation have been reported (Osorio *et al.*, 2016). However, to our knowledge, no studies have incorporated the spatial analysis of the ocurrence of drugs of abuse–population characteristics in river basins.

The aim of this study is to establish the influence and the effects of the human pressure in a typical Mediterranean river basin (Turia River) through the analysis of the occurrence and distribution of drugs of abuse. Combining these data and several basic characteristics (population size and distribution, location of wastewater treatment plants (WWTP), etc.) through the geographical information system (GIS), the suitable range of each factor affecting attenuation and spatial distribution of these compounds were assessed (Pascual-Aguilar *et al.*, 2015; Pascual-Aguilar *et al.*, 2013; Terrado *et al.*, 2006). Furthermore, the statistical relation between presence of drugs of abuse and physco-chemical characteristics of water was statistically studied. The environmental hazard of drugs of abuse was assessed using the available data on toxicity. This study is aimed to create an essential knowledge base for the development, from a holistic perspective, of more effective and reliable policies of Mediterranean river basins recovery and protection, which can be potentially of help to preserve public health and safety.

2. Experimental

2.1 Reagents and materials

High purity (>99%) standard solutions of 42 drugs of abuse and metabolites and 18 deuterated analogues were purchased from Cerillant (Austin, TX, USA) and LGC GMBH

(Luckenwalde, Germany). Deuterated compounds were used as internal standards (IS). **Table S8.1** in the supplementary content summarizes the abbreviations and chemical properties of all the compounds. Working standard solutions were prepared at different concentrations by appropriate dilution of the individual stock solutions in methanol-water (1:9, v/v). Calibration standards were prepared by serial dilution of the mixed working solution. Stock and working solutions were stored at -20ºC in the dark. Water used for preparation of calibration standards and LC-MS mobile phase was purified by an Elix Milli-Q system (Millipore, Billerica, MA, USA). Methanol was purchased from Panreac (Castellar del Vallès, Barcelona, Spain) and formic acid was purchased from Amresco (Solon, OH, USA).

2.2 Study area

The Turia River is 280 km length with an average flow rate of 10.43 m^3/s and a total drainage area of 6393.6 km^2 that flows into the Mediterranean Sea in the city of Valencia, Spain. It receives as the most important tributary the river Alfambra (with 60 km, 1398 km^2 and 1.5 m^3/s) (Ccanccapa *et al.*, 2016). The annual average rainfall in the Jucar Hidrographic Confederation, where the Turia River belongs, was 445 mm in 2012 and 507 mm in 2013. These data were lower than those reported for the total rainfall in the Iberian Peninsula in these years, 570 and 770 mm respectively. The rainy season in the Turia River basin is from September to May, with maximum rainfall in November in 2012 (105 mm) and April in 2013 (100 mm) but there is a large spatial variation. Specifically, the average rainfall during the sampling period were 12.5 and 22.5 in 2012 and 2013, respectively (EDADES, 2013; IAEST, 2012). The annual average temperature presents huge oscillations depending on the localization along the Turia River basin. The temperature in the upper basin oscillated between 1.9 and 24.3 with an annual average

temperature of 12.9 ºC in 2012 and between 3.7 and 23 ºC with an annual average temperature of 12.1 ºC in 2013. On the other hand, in the medium and lower basin the annual average temperature ranged from 12.4 to 26.0 ºC in 2013 and from 9.8 to 27.4 ºC, with an average temperature in both years of 18.7. The highest temperatures in all cases were reached in June, July and August (INE, 2012, 2013).

2.3 Sampling and sample analysis

Sampling campaigns were performed during 2012 and 2013 along the Turia River. **Figure S8.1** shows the location of the different sampling points in Turia River basin. Sampling locations were homogenously distributed through the course of the river from its head to its mouth. The monitoring was carried out in October 2012 and July 2013. Surface water samples were collected from 22 sampling sites in 2012 and 31 in 2013 distributed along the river. Grab water samples (2 L) were gathered in amber glass bottles and transported in hermetic refrigerated boxes with ice upon arrival at the laboratory. Physical and chemical characteristics of water (temperature, pH, redox potential (mV), conductivity (Cond), total dissolved salts (TDS), resistivity (Res) and dissolved oxygen (DO) were recorded at the sampling sites using a Multiparameter Eutech Instrument CyberScan PCD 650 (Thermo Fisher Scientific, Basel, Switzerland). Once the samples arrived to the laboratory were immediately frozen at -20ºC until analysis to prevent degradation of the psychoactive compounds, and were analysed in the 5 subsequent days.

Analysis of the target illicit drugs was performed following a previous method based on solid phase extraction and liquid chromatography coupled mass spectrometry (SPE-LC-MS/MS) (Álvarez-Ruiz *et al.*, 2015; Andrés-Costa *et al.*, 2014). Psychoactive substances were extracted from water by SPE using Phenomenex Strata-X cartridges (Torrance, Ca, USA). Analytes were eluted with 6 mL of methanol and 3 mL of methanol-

dichloromethane (1:1, v/v), evaporated to dryness and redissolved in 1 mL of water-methanol (9:1, v/v). The LC-MS/MS was performed using an Agilent 1260 UHPLC coupled to an Agilent 6410 triple quadrupole mass spectrometer with an electrospray ionization source working in the positive ionization (ESI+) mode. Details on the experimental conditions and the performance of the analytical method applied are summarized in the supplementary content (**Table S8.2, S8.3** and **S8.4**).

2.4 GIS

Spatial distribution of all information gathered was performed using GIS techniques with ARCGIS (V. 10.1). Information obtained by different means (census data, fieldwork, new GIS layers) was integrated into a common framework that could explain the spatial representativeness of anthropogenic pressures on incoming surface waters of the Turia River basin.

The analysis was performed following and environmental forensic perspective and was organized in two major steps. First, all data were integrated into a common GIS analytical structure including a point layer with the location of WWTP, a point layer with the location and analytical values of the points with information of contaminants, a line layer with the river network and a polygon map (municipal division) with statistical data on population divided by sex and different ranges of age. GIS covers where projected and georeferenced (reference system ETRS-1989) according to official standards following the Spanish Spatial Data Infrastructure.

The second step consisted on the spatial analysis of drugs of abuse. A descriptive model of territorial presence of contaminants were stablished considering the combination of the location of contaminants at a particular place with population densities. Particular attention was paid to population between age 15 and 64, matching the age ranges

established in epidemiologic studies of drug consumption from surveys in Spain (EDADES, 2013).

2.5 Source of data and Statistical analysis

Different data were used to carry out this study. Municipal statistical values on the number of inhabitants for the years 2012 and 2013 were provided by Spanish Institute of Statistics and La Caixa (Caixabank; INE., 2014) . Climatic conditions, flow rate and other physical parameters of the sampling campaigns were obtained from Jucar Hidrographic Confederation database (CHJ, 2012-2013).

IBM SPSS v. 22.0 (SPSS Inc., USA) was used for statistical analyses including principal component analysis (PCA). Analysis of variance (ANOVA) and Tukey's multiple range test at $\alpha = 0.05$ were performed to detect differences in the variables. In the cases where the homogeneity and/or normality of the data could not be assumed, the Kruskal-Wallis and Mann-Whitney non parametric test ($P \leq 0.05$) were applied. Pearson statistical bivariate correlation analyses were implemented, at 95% and 99% significance levels, between drugs of abuse concentrations and water intrinsic quality parameters and rainfall events to determine possible relationships among them. When the values of a variable showed a non-normal distribution, Spearman bivariate correlations were applied at the same significance levels. Multiple stepwise linear regression analysis and categorical PCA were used to confirm the weight and dependence between variables differences and identifying patterns in them.

2.6 Ecotoxicological analysis

Toxicity data (EC_{50} or LC_{50}) was collected after literature review and using ECOSAR for those drugs of abuse that there was no experimental toxicity data in

literature. This program is widely used to predict the toxicity of determined compounds under aqueous conditions, based on the similarity of structure to other compounds whose toxicity in aquatic environment has been previouly estimated (Andrés-Costa *et al.*, 2014; Gros *et al.*, 2010; Thomaidi *et al.*, 2015).

RQs were calculated for detected drugs of abuse in Turia River, for 3 different aquatic organisms (fish, daphnia magna, algae). For target compounds where more than one toxicity data was available, the lowest value was chosen in order to estimate ecological threat for worst-case scenario. The RQs for the individual compounds were calculated using Eq. (1):

$$RQ = \frac{MEC}{PNEC}$$ Equation (8.1)

where MEC is the measured environmental concentration and PNEC is the predicted no effect concentration, PNEC was calculated from EC_{50} or LC_{50}. According to the Technical Guidance Document of the European Commision (Commission, 2003) , PNEC was calculated by dividing the LC_{50} or EC_{50} value by an appropiate assessment factor (AF). Since only short-term toxicity data were available, an AF of 1000 was applied on the lowest LC_{50} or EC_{50} value (Eq. 2).

$$PNEC = \frac{LC_{50} \text{ or } EC_{50}}{AF}$$ Equation (8.2)

There are different risk levels regarding RQ values, low risk for RQ less than 0.1, medium risk for RQ between 0.1 and 1 and high risk for RQ greater than 1 (Hernando *et al.*, 2006).

3. Results

3.1 Ocurrence of drugs of abuse

Table 8.1 summarizes the concentration levels (minimum, maximum and mean) and frequency of detection of the studied drugs of abuse and metabolites in the surface

water samples analysed from the Turia River basin in both campaigns (**Table S8.5** in supplementary content depicts detailed concentrations of positive drugs in all sampling points).

The presence of drugs of abuse was scarce in surface waters, probably because these compounds were easily degraded. In 2012, 6 psychoactive substances (MDMA, PMA, BUF, 4-MeO-PCP, BECG and MET) were detected in 22 sampling points. In 2013, 7 compounds (EPH, MDMA, 4-MeO-PCP, BECG, ECME, COD and MET) were detected in a total of 31 sampling sites, 4 of them (MDMA, 4-MeO-PCP, BECG and MET) were also detected in 2012.

According to their occurrence, drugs of abuse can be clasified in three groups:

(i) Drugs detected only in one sampling point. In 2012, MDMA and 4-MeO-PCP were detected at a concentration of 22.77 and 37.61 ng/L, respectively. In 2013, 4-MeO-PCP was detected in a different sampling point of 2012 at a concentration of 7.55 ng/L and ECME was detected at a concentration of 15.03 ng/L.

(ii) Drugs detected only in a few sampling points. BUF, MET and PMA were found out in 3 or 4 sampling points at concentrations <70 ng/L in 2012. EPH and COD were detected in 3 sampling point at average concentrations of 11.60 ng/L for EPH and 91.31 ng/L for COD in 2013.

(iii) Drugs detected in more than 5 sampling points. The compound detected more frequently along the river was BECG, a COC metabolite, in a mean concentration of 25.45 (2.91 – 76.76) ng/L. In 2013, MDMA was detected in 5 sampling points (mean of 4.67 ng/L, ranged from 2.34 to 7.21 ng/L) and BECG and MET were detected in a total of 8 and 7 sampling points, respectively, each one at a mean concentration of 14.02 (1.83 – 12.75) ng/L for BECG and 11.42 (2.29 – 40.07) ng/L for MET.

The concentration of BECG was similar to concentrations found in other Spanish rivers as Llobregat (7 – 12 ng/L), Guadalquivir (16.3, 5.6 – 9.8 ng/L) and Tajo (29.2 ng/L). However, it was lower than those reported in Jarama and Manzanares (119 – 145 ng/L) (Mendoza, López de Alda*, et al.*, 2014; R. Pal *et al.*, 2013). At the European level, our results agree with those carried out in Po and Arno River (25 and 21.8 ng/L, respectevily) in Italy and in Ely (12 ng/L) and Calder (26.8 ng/L) rivers in UK (Baker *et al.*, 2011; R. Pal *et al.*, 2013). Only van Nuijs et al., 2009 found ECME at a concentration below LOQ (20 ng/L), probably because of its low stability in water and its high LOQ compared to BECG and COC (van Nuijs *et al.*, 2009).

The concentration of MET was higher than those reported in Olona, Lambro and Arno (8.6, 3.4 and 4.8 ng/L) in Italy (R. Pal *et al.*, 2013), in Calder (10 ng/L) in UK (Baker *et al.*, 2011) and in Llobregat, Henares and Tajo (6.4, 7 and 2.6 ng/L) in Spain (R. Pal *et al.*, 2013) and lower than those reported in Jarama and Manzanares (25.6 – 37 ng/L) rivers also in Spain (Mendoza, López de Alda*, et al.*, 2014).

The concentrations of COD were in agreement with those reported by Baker et al., 2011 in Calder river (128.2 ng/L) in UK (Baker *et al.*, 2011) and higher than those reported in 22 fluvio-litoral systems (2.9 ng/L) in Switzerland (Berset *et al.*, 2010) and in the Guadalquivir River (40.4 ng/L) in Spain (Robles-Molina *et al.*, 2014).

The concentration of MDMA was similar to the concentrations reported by Mendoza et al., 2014 in Jarama and Manzanares (10.2 – 25.7 ng/L) in Spain, by Baker and Kasprzyk-Hordern, 2011 in Calder (8.7 ng/L) in UK and by Senta et al., 2015 in Sava (8.6 ng/L) in Croatia and higher than concentrations reported by Mastroianni et al., 2016 in LLobregat, Ebro, Jucar and Guadalquivir (1.3 – 2.8, 0.4 – 0.6, 0.3 – 0.5, 0.9 – 1.0 ng/L) in Spain.

The concentrations of EPH determined in the present study were similar to those determined in Guadalquivir (7.2 ng/L, 14.5 ng/L) and in Llobregat (11.8 ng/L) (Mastroianni *et al.*, 2016; Robles-Molina *et al.*, 2014) in Spain and lower than concentrations reported in Jarama and Manzanares (204 – 206 ng/L) in Spain.

PMA, BUF and 4-MeO-PCP were not reported previously in river water samples. Being this study that reports their first occurrence in environmental waters.

3.2 Water quality data sources

The temperature, pH, Cond, Res, TDS,DO and rainfall are presented in **Table 8.2** as mean values ± SD (specific values for each sampling point are depicted in **Table S8.6** in the supplementary content). These environmental conditions might have an effect in levels of drugs of abuse along the Turia River. Values of river water temperatures ranged from 10.5 to 30.0 ºC in both campaings, being average temperatures in 2012 (October) moderetaly lower than 2013 (July). Water pH values in all sampling points were slightly basic (7.89 – 9.14). Temperature and pH are parameters that influence chemical and biochemical reactions and there are several studies that reported the effectes of these variables on the stability of drugs of abuse in water matrices. Stability of cocainics has been studied over a range of different conditions (McCall *et al.*, 2016). Hydrolysis of COC seems pH-dependent, being neutral and basic pH that cause greater degradation (Warner *et al.*, 2000), in fact, COC was not detected in this study, probably because its low stability in water. Gheorge et al., (2008) studied pH and temperature stability of cocainics evidencing BE stability for 5 days at pH values of 2 and 6 and three temperatures (-20ºC, 4ºC and 20ºC). However, a dramatic degradation of COC and ECME was observed at pH 6 and temperature of 20ºC during 24 h (Gheorghe *et al.*, 2008). Several studies demonstrated amphetamine-

type substances have shown high stability (< 10% transformation) at pH 7 and 20ºC for 24 h (McCall *et al.*, 2016). Opiates show variable stability, for example HER and 6-MAM showed low stability in water at 20 ºC while METH, EDDP and COD seem to be stable at 19 ºC and pH 7. Stability of cannabinoids is variable depending the study but Senta et al. (2014) present < 20% transformation at pH 7.4 and 20ºC (Senta *et al.*, 2014). TDS (124.8 – 2291.0 ppm), conductivity (0.68 – 1.67 dS/m) and resistivity (218.2 – 3973.0 Ω) are parameters responsible for establishing salinity. The values of these parameters worsen from the source to the mouth of the Turia River, pointing out worse quality of water in the source, where there are major presence of industrial, agricultural and urbanizated zones. DO (5.14 – 12.78 mg/L) is an important parameter in assessing water quality because of its influence on the organisms living within a body of water, so lowest values for DO, as P5 (6.72 mg/L) and P20 (6.28 mg/L) in 2012 and P34 (5.14 mg/L) in 2013, indicate contamination, septic conditions of organic matter or an intense bacterial activity.

Rainfalls during sampling campaigns were recorded (**Table S8.6** in supplementary content). In 2012 there were less rainfall events in sampling days (0.65 L/m^2) than in 2013 (1.85 L/m^2). Regarding accumulated and average rainfalls, in 2012 the accumulated (44.03 L/m^2) and average (2.94 L/m^2) rainfalls during the 15 days before the sampling day were higher than in 2013 with 13.26 and 0.88 L/m^2, respectively.

3.3 Statistic analysis

Significant correlations were found between COD and MDMA, EPH or MET, 4-MeO-PCP and MDMA as well as BECG and BUF at 99% of probability. In addition, correlation were also found between EPH and MDMA, MET and EPH or MDMA as

well as BECG and COD at 95% (see **Table S8.7** in supplementary content). COD, EPH and MET are also used in hospitals for different purposed. 4-MeO-PCP and MDMA are both new design drugs.

Several significant correlations between drugs of abuse and quality parameters of surface water and rainfall events were found. **Table S8.8** in supplementary content shows correlations between temperature and EPH or COD, TDS and EPH or COD, Res and EPH or COD, as well as average of rainfall (15 days) and MDMA or COD at 99%. Correlation between temperature and MDMA, Cond and EPH, BECG or COD, TDS and MDMA, Res and MDMA, DO and EPH, COD or MET, rainfall (sampling day) and ECME, as well as average of rainfall (15 days) and EPH were also found at 95%. The relationship between drugs of abuse concentrations and quality parameters and rainfall events was investigated by multiple stepwise linear regression and results are presented in **Tables S8.9** and **S8.10** in supplementary content. Regarding detected concentrations of drugs of abuse EPH, MDMA and MET are correlated with COD. Furthermore, 4-MeO-PCP and COD are connected to MDMA and BECG. Temperature, Cond and DO are the quality parameters that showed more influence on detected drugs of abuse. Specifically, EPH and COD be affected by temperature and BECG and MET are influenced by Cond and DO, respectively. Rainfall events affect as average rainfall during 15 days before sampling to MDMA and rainfall of sampling day to ECME.

Drugs of abuse data set was subjected to PCA to confirm the compositional and similarities of drugs of abuse and quality parameters in Turia River surface water. **Figure 8.1** depicts one clear cluster with certain drugs of abuse as COD, MDMA, EPH and quality parameters of Cond and temperature.

3.4 GIS

The low frequency of occurrence of drugs of abuse makes complicate to relate the patterns of distribution of these compounds to the characteristics of the basin or population in a qualitative way. Spatial distribution of drugs of abuse was established considering the combination of the occurrence of contaminants with locations of WWTP and statistical data of population. Even though, insufficiently treated municipal WWTP discharge is identified as the route responsible for surface water contamination, there was no significant trend between the location of WWTP and the presence of drugs of abuse. A possible reason could be the low half-life and stability of these drugs in water and the presence of other contamination sources as uncontrolled discharges into the river. Regarding the population statistical data, several statistic indicators were used: (i) density and ratio of young population (15 – 34 years), (ii) density and ratio of general population (15 – 64 years), (iii) density and ration of women population (15 – 64 years) and (iv) density and ratio of men population. According to the survey of alcohol and drugs of abuse in Spain (EDADES, 2013), in general, the consumption of legal drugs is higher by older population (35 – 64 years), while illegal ones as MDMA, amphetamines, cocainics and cannabis have a greater impact on the young population (15 – 34 years). Regarding the prevalence of psychoactive substances according to sex, it was observed that women only exceed the consumption of men for hypnotics (mostly tranquilizers). However, in this study no significant correlations between density and ratio of young population and sex were found. To establish any of the mentioned correlations a deeper study that involve more samples taken at different season of the year would be required.

The statistical indicator that provided a significative trend with presence of drugs of abuse along Turia River basin was the density of population between 15 and 64 years. The distribution of drugs of abuse in Turia River in 2012 and 2013 is represented

in **Figure 8.2** and **8.3**, respectively. The highest concentrations and frequency of drugs of abuse appears in the sampling points corresponding to areas with highest population density. This relation between presence of drugs of abuse and high density population can explain why in the comparison of the results of our study with other reported in Spain the highest levels were always observed in the area of Manzares/Jarama, which sorrounds Madrid, the largest city in Spain (see section 3.1).

3.5 Ecological assessment

The RQ has become a parameter to characterize ecological risk of emerging contaminants. If RQ is higher or equal to 1, it suggests that this compound could cause potentially adverse ecological effects (Thomaidi *et al.*, 2015). **Table 8.3** summarizes RQ values for (a) fish, (b) daphnids and (c) green algae. Acute toxicity values were used to calculate the PNEC for each detected drugs of abuse in river water. According to RQ classification (low risk from 0.01 to 0.1, medium risk from 0.1 to 1 and high risk >1), compounds detected in Turia river do not pose acute risks to aquatic organisms as their RQ values were lower than 1. However, emerging contaminants are persistent or pseudo-persistent in the environment, so chronic exposure to these compounds could produce long-term toxicity effects. The information on ecotoxicity of drugs of abuse in scientific literature is limited and not systematic, and the most available information is theorical. Mastroianni et al. (2016) indicate that EDDP, METH and MDMA showed cumulative RQ > 1 in 4 samples in surface waters (Mastroianni *et al.*, 2016). The RQ values for METH, AMP, KET and EPH were between 0.00 and 0.05, suggesting that adverse effects were improbable (Zhang *et al.*, 2017). The environmental risk of drugs of abuse as RQ of MAMP, MDMA, COC, BECG, MOR and COD estimated by Van der Aa et al., 2013 were lower than 1 (van der Aa *et al.*, 2013). Furtheremore, in a Mediterranean river (Llobregat, NE Spain), the RQ values for the illicit drugs were

below 1 (Lopez-Serna *et al.*, 2012). Our results agree with previous studies indicating low or no risk to the acuatic environment.

Limitations of this risk assessment are (i) most important the lack of experimental toxicity data, then quantitative structure–activity relationship (QSAR) by ECOSAR was used, and (ii) occurrence of these compounds was estimated from grab samples which may not be representative of a long term exposure. Before discarding these compounds ecotoxicological risk, more ecotoxicological data, especially on cronic exposure would be required. A relevant ecotoxicological characterization of these drugs of abuse would require the systematic implementation of a complete battery of bioassays using ecologically relevant organisms and biologically relevant endpoints.

Conclusions

On the occurrence of drugs of abuse (frequency and concentration), the most ubiquitous drug of abuse detected along the Turia River in both campaigns was BECG, detected in 9 and 8 sampling sites in 2012 and 2013, respectively. This study describes the first occurrence of PMA, BUF and 4-MeO-PCP in surface waters. The occurrence of these drugs is higher near of the city with highest population densities according to the descriptive model of territorial presence. Compounds used as drugs of abuse and prescribed pharmaceuticals (MET, COD and EPH) were mostly detected in Valencia city where most hospitals are located. The highest detected concentration belongs to COD. However, relationships are difficult to establish because drugs of abuse are pseudo-persistent contaminants (high transformation rates are compensated by their continuous introduction in the environment). The effects of other parameters as the influence of water quality (worst in highly populated areas) in the transformation rates of these compounds needs to be further studied because many illicit drugs are quite

unstable. Although risk assesment showed low ecotoxicological harzard, further studies are also needed in order to assess long term toxicity.

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Table 8.1. Concentration (mean, minimum and maximum) and frequency of illicit drugs detected in Turia River in 2012 and 2013 campaigns.

Illicit drugs	2012			2013		
	Concentration (ng/L)			Concentration (ng/L)		
	Mean	Min-Max	[a]Freq (22)	Mean	Min-Max	[b]Freq (31)
AMP	n.d.			n.d.		
MAMP	n.d.			n.d.		
ETAMINE	n.d.			n.d.		
EPH	n.d.			11.60	5.28 - 17.65	3
EPHED	n.a.			n.d.		
MEPHEN	n.d.			n.d.		
MEP	n.d.			n.d.		
METONE	n.d.			n.d.		
MDMA	22.77	22.77 – 22.77	1	4.67	2.34 - 7.21	5
MDA	n.d.			n.d.		
MDEA	n.d.			n.d.		
MBDB	n.d.			n.d.		
bk-MMBDB	n.d.			n.d.		
2C-B	n.d.			n.d.		
NAPH	n.d.			n.d.		
MDPV	n.d.			n.d.		
PMA	12.34	5.11-19.25	4	n.d.		
4-AcO-DIPT	n.a.			n.d.		
BUF	29.33	9.67 – 66.77	3	n.d.		
mCPP	n.d.			n.d.		
TFMPP	n.d.			n.d.		
PPP	n.d.			n.d.		
α-PVP	n.d.			n.d.		
MDPPP	n.a.			n.d.		
4-MePPP	n.a.			n.d.		
4'MePHP	n.d.			n.d.		
MPBP	n.d.			n.d.		
4-MeO-PCP	37.61	37.61 – 37.61	1	7.55	7.55 – 7.55	1
KET	n.d.			n.d.		
COC	n.d.			n.d.		
BECG	25.45	2.91 – 76.76	9	14.02	1.83 – 12.75	8
COCET	n.d.			n.d.		
ECME	n.d.			15.03	15.03 – 15.03	1
6-MAM	n.d.			n.d.		
COD	n.a			91.31	81.52 - 101.02	3
EDDP	n.d.			n.d.		
HER	n.d.			n.d.		
MET	15.20	2.02 – 39.29	3	11.42	2.29 – 40.07	7
MOR	n.d.			n.d.		
JWH-018	n.d.			n.d.		
THC	n.d.			n.d.		
THC-COOH	n.d.			n.d.		

Min: minimum; Max: maximum; Freq: frequency; n.d.: non detected; n.a.: not analyzed
[a,b] Positive sampling points regarding the total sampling points analysed in each campaign

Table 8.2. Mean, minimum and maximum values of physico-chemical parameters in in Turia River samples in 2012 and 2013 campaigns.

		2012		2013	
		Mean ± SD	Min-Max	Mean ± SD	Min-Max
Temperature (ºC)		14.55 ± 3.07	10.5 – 20.2	19.46 ± 4.48	12.8 – 30.00
pH		8.28 ± 0.18	7.89 – 8.75	8.62 ± 0.38	7.50 – 9.14
mV		-68.55 ± 8.19	- 82.9 – - 47.8	-88.20 ± 4.48	-117.00 – -29.60
Cond (dS/m)		0.99 ± 0.23	0.68 – 1.38	0.92 ± 0.32	0.17 – 1.67
TDS (ppm)		667.79 ± 230.16	366.30 – 1045.00	850.44 ± 472.73	124.80 – 2291.00
Res (Ω)		847.50 ± 298.32	480.20 – 1365.00	825.14 ± 684.76	218.20 – 3973.00
DO (mg/L)		5.60 ± 1.13	6.28 – 10.22	9.70 ± 1.71	5.14 – 12.78
	sampling day	0.65 ± 2.23	0.00 – 9.85	1.84 ± 2.75	0.00 – 7.80
Rainfall (L/ m^2)	15 days accumulated	44.03 ± 27.96	0.20 – 92.60	13.26 ± 16.05	0.00 – 49.00
	15 days average	2.94 ± 1.86	0.07 – 6.17	0.88 ± 1.07	0.00 – 3.27

SD: standard deviation; Cond: conductivity; TDS: total dissolved salts; Res: resistivity; DO: dissolved oxygen

Table 8.3. Estimation of Risk Quotients, RQ (MEC/PNEC) for drugs of abuse detected in Turia River in 2012 and 2013 campaigns.

	RQ 2012			RQ 2013		
	Fish	Dapnids	Green algae	Fish	Dapnids	Green algae
EPH	0.00	0.00	0.00	0.00	0.00	0.00
MDMA	0.11	0.11	0.00	0.04	0.04	0.00
PMA	0.00	0.00	0.00	0.00	0.00	0.00
BUF	0.26	0.26	0.01	0.00	0.00	0.00
4-MeO-PCP	0.38	0.38	0.92	0.08	0.08	0.18
BECG	0.00	0.00	0.00	0.00	0.00	0.00
ECME	0.00	0.00	0.00	0.00	0.00	0.00
COD	0.00	0.00	0.00	0.10	0.10	0.01
MET	0.11	0.11	0.23	0.12	0.12	0.23

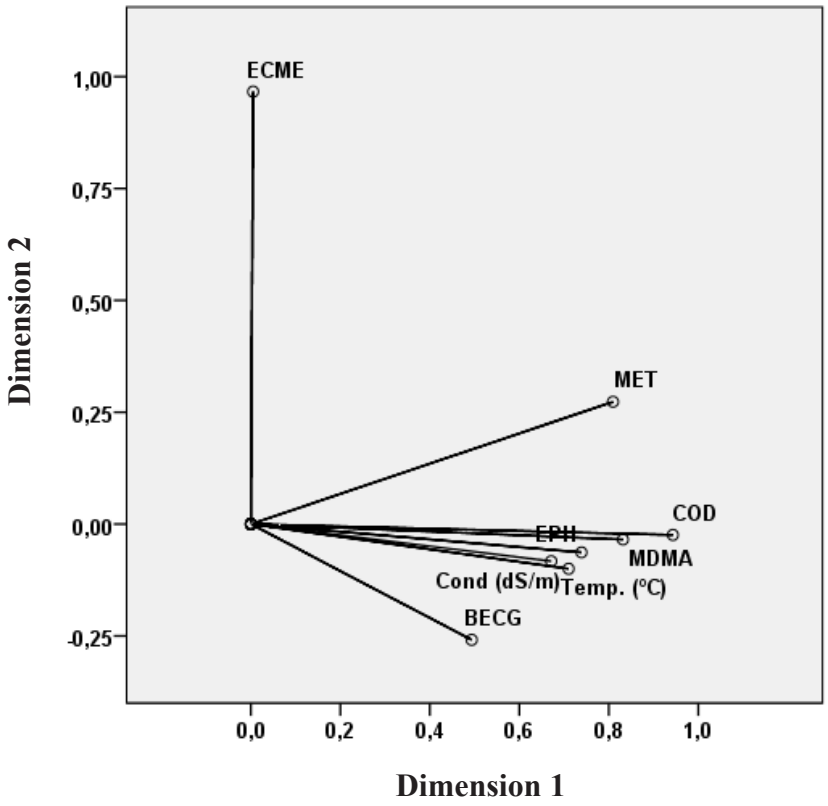

Figure 8.1. PCA analysis of the data of Turia River

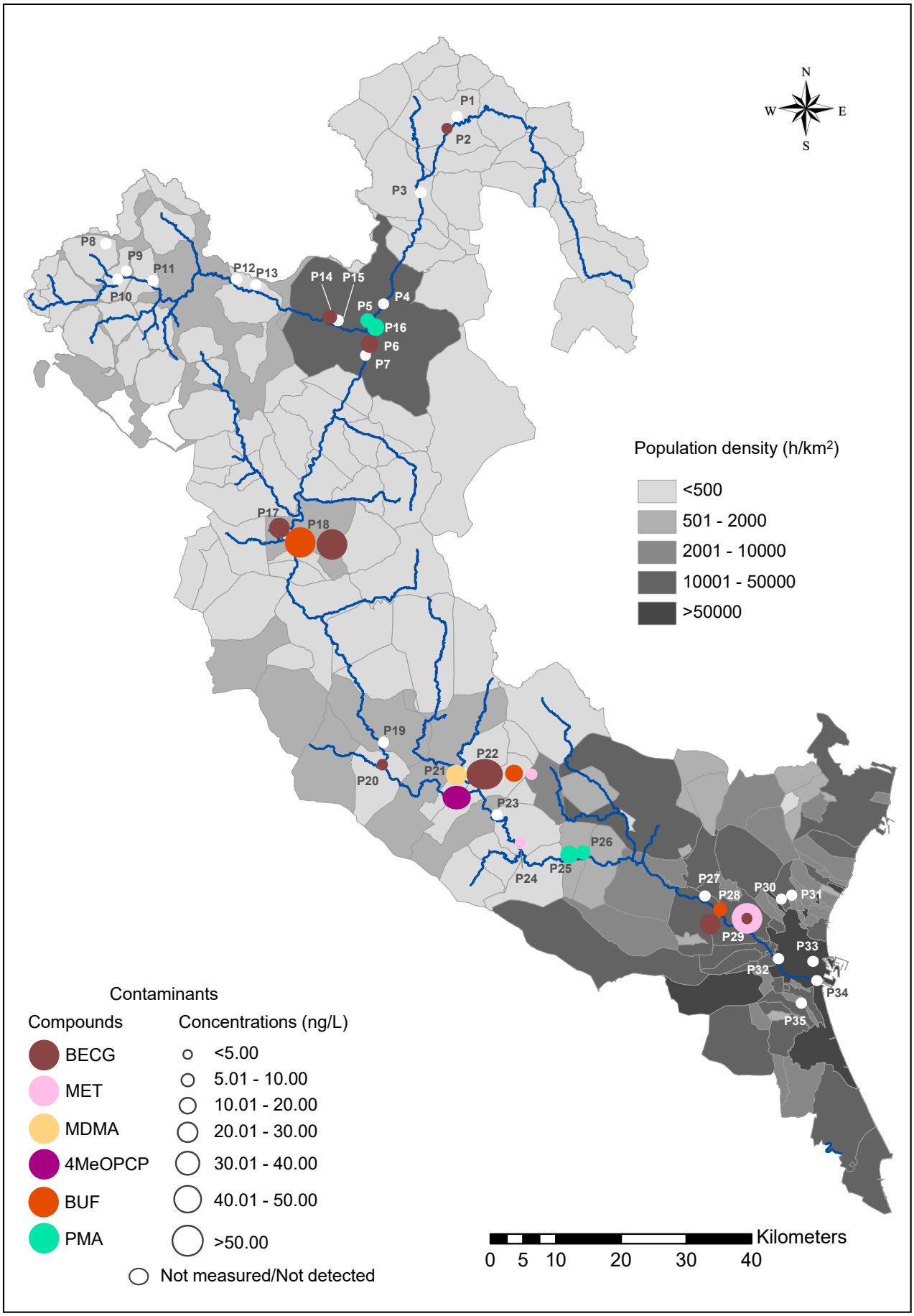

Figure 8.2. Distribution pattern of drugs of abuse and density population (15 – 64 years) in the Turia River in 2012 by GIS

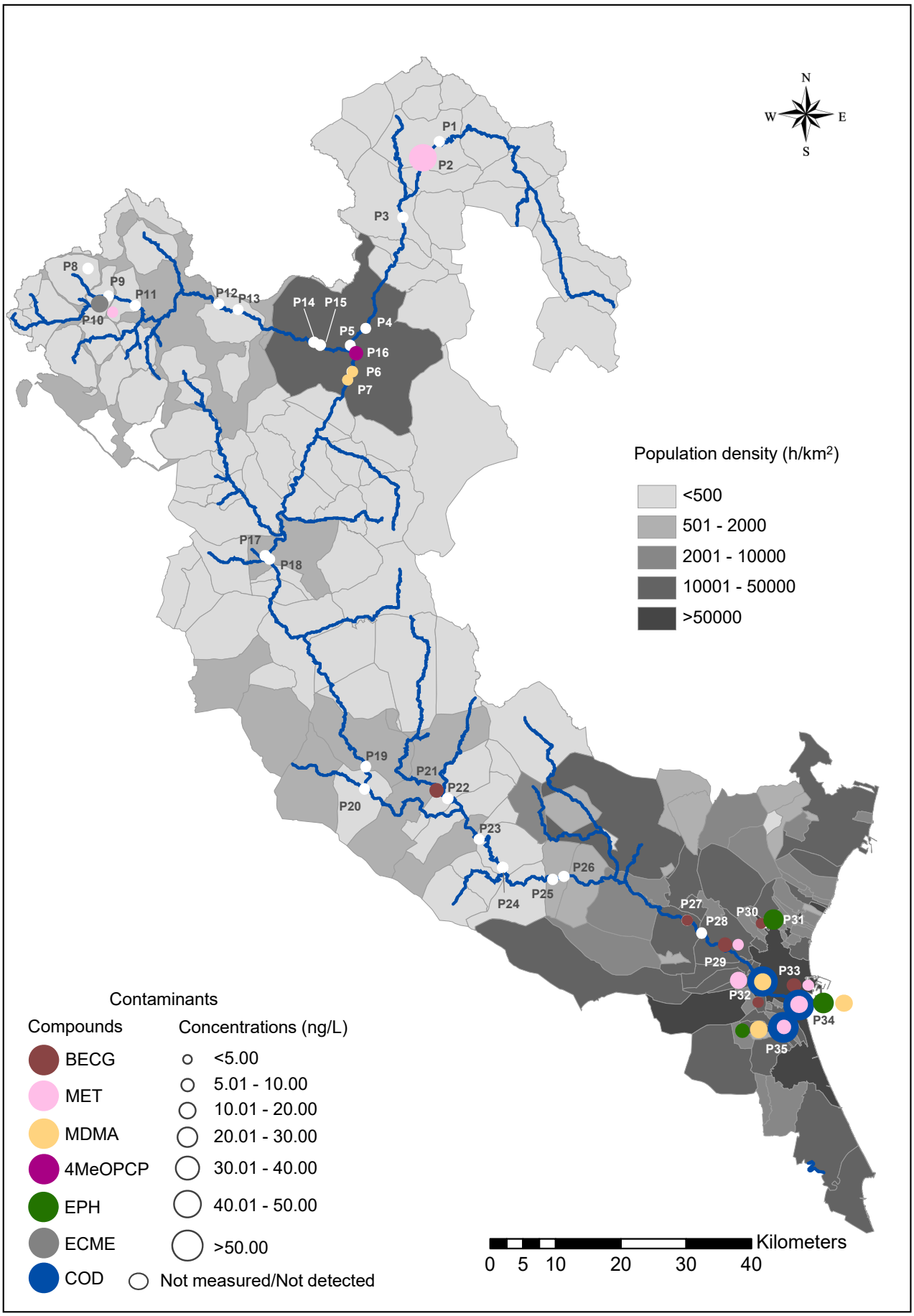

Figure 8.3. Distribution pattern of drugs of abuse and density population (15 – 64 years) in the Turia River in 2013 by GIS

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SUPPLEMENTARY CONTENT

ASSESSING DRUGS OF ABUSE DISTRIBUITON IN TURIA RIVER BASED ON GEOGRAPHIC INFORMATION SYSTEM AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

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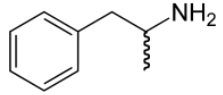
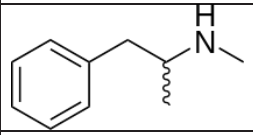
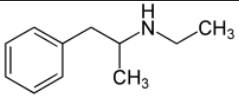
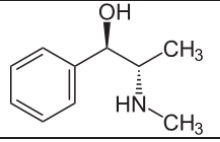
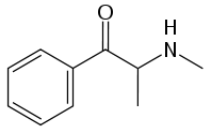
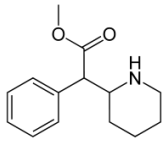
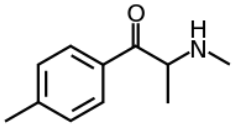
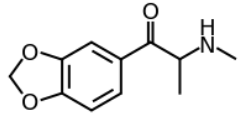
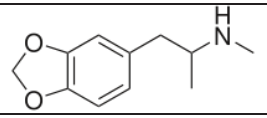
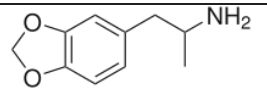
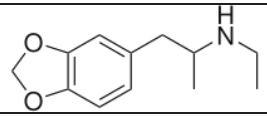
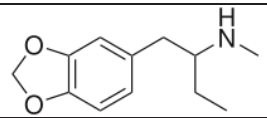
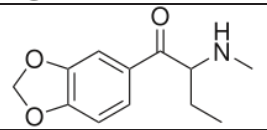
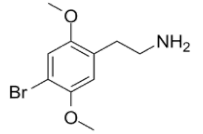
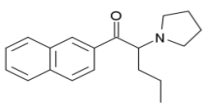
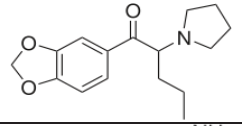
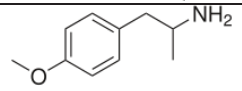
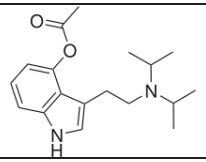
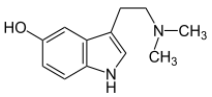
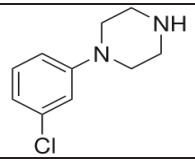
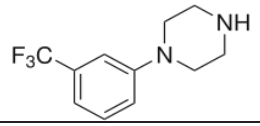
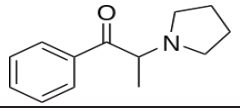
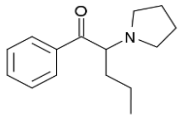
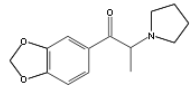
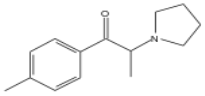
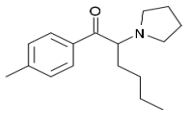
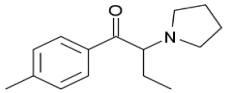
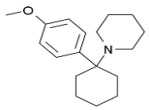
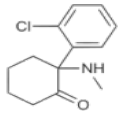
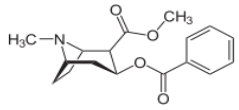
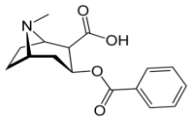
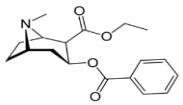
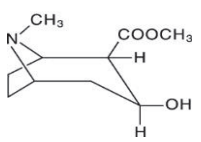
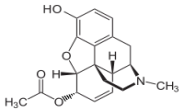
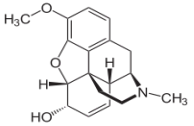
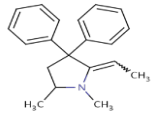
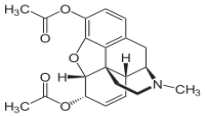
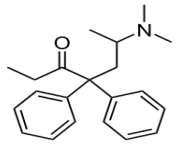
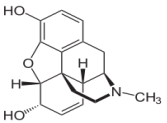
Table S8.1. Name, abbreviation, empirical formula, structure, molecular weight, CAS number, log P, pKa of psychoactive substances.

Name	Abbreviation	Empirical Formula	Structure	MW (g mol^{-1})	CAS Number	Log P	pKa
AMPHETAMINES							
Amphetamine	AMP[1]	C9H13N		135.2062 (A) 135.104799421 (M)	300-62-9	1.76	10.01
	AMP-d5	C9H8D5N					
Methamphetamine	MAMP[2]	C10H15N		149.2328 (A) 149.120449 (M)	537-46-2	2.07	10.21
	MAMP-d5	C10H10D5N					
Ethylamphetamine	ETAMINE[4]	C11H17N		207.2689 (A) 207.125928 (M)	14089-52-2	2.25	10.22
Ephedrine	EPH[1]	C10H15NO		165.2322 (A) 165.115364 (M)	299-42-3	1.13	9.52
Ephedrone	EPHED[1]	C10H13NO		163.21632 (A) 163.099714 (M)	5650-44-2	1.61	8.02
Methylphenidate	MEPHEN[2]	C14H19NO2		233.3062 (A) 233.141578857 (M)	113-45-1	2.25	9.09
Mephedrone	MEP[2]	C11H15NO		177.2429 (A) 177.115364 (M)	1189805-46-6	2.12	8.05
Methylone	METONE[3]	C11H13NO3		207.22582 (A) 207.089543 (M)	186028-79-5	1.23	7.90

	METONE-d3	C11H10D3NO3					
3,4-methylenedioxymethamphetamine	MDMA[4]	C11H15NO2		193.2423 (A) 193.110275 (M)	42542-10-9	1.86	10.14
	MDMA-d5	C11H10D5NO2					
3,4-methylendioxyamphetamine	MDA[5]	C10H13NO2		179.2157 (A) 179.094628665 (M)	4764-17-4	1.43	10.01
	MDA-d5	C10H8D5NO2					
3,4-methylenedioxy-N-ethylamphetamine	MDEA[6]	C12H17NO2		207.2689 (A) 207.125928793 (M)	14089-52-2	2.22	10.22
	MDEA-d5	C12H12D5NO2					
N-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine	MBDB[4]	C12H17NO2		207.26888 (A) 207.125929 (M)	103818-46-8	2.38	10.30
Dibutylone	bk-MMBDB[4]	C13H17NO3		235.27898 (A) 235.120843 (M)	802286-83-5	2.14	7.10
4-Bromo-2,5-dimethoxyphenethylamine	2C-B[4]	C10H14BrNO2		260.128 (A) 259.020791344 (M)	66142-81-2	1.84	9.68
Naphyrone	NAPH[3]	C19H23NO		281.39202 (A) 281.177964 (M)	850352-53-3	4.35	8.00
3,4-methylenedioxypyrovalerone	MDPV[4]	C16H21NO3		275.34284 (A) 275.152144 (M)	687603-66-3	2.99	7.4
para-methoxyamphetamine	PMA[3]	C10H15NO		165.2322 (A) 165.115364107 (M)	23239-32-9	1.65	10.00

TRYPTAMINES							
4-acetoxy-N,N-dimethyltryptamine	4-AcO-DIPT[4]	C18H26N2O2		302.4112 (A) 302.199428086 (M)	936015-60-0	3.46	10.6
Bufotenine	BUF[4]	C12H16N2O		204.2682 (A) 204.126263144 (M)	487-93-4	1.29	9.91
PIPERAZINES							
1-(3-chlorophenil)piperazine	mCPP[4]	C10H13ClN2		196.677 (A) 196.076726133 (M)	6640-24-0	2.15	8.87
1-(3-trifluoromethylphenyl)piperazine	TFMPP[4]	C11H13F3N2		230.2295 (A) 230.103083041 (M)	239-574-4	2.42	8.90
PYRROLIDINOPHENONE							
α-pyrrolidinopropiophenone	PPP[4]	C13H17NO		203.2802 (A) 203.131014171 (M)	19134-50-0	2.40	7.40
α-pyrrolidinovalerophenone	α-PVP[4]	C15H21NO		231.3333 (A) 231.162314299 (M)	14530-33-7	3.36	7.90
3',4'-Methylenedioxy-α-pyrrolidinopropiophenone	MDPPP[4]	C14H17NO3		247.2897 (A) 247.120843415 (M)	24698-57-5	2.02	6.80
4-methyl-α-pyrrolidinopropiophenone	4-MePPP[4]	C14H19NO		217.3068 (A) 217.146664235 (M)	28117-80-8	2.91	7.50

4-methyl-α-pyrrolidinohexaphenone	4'-MePHP[4]	C17H25NO		259.3865 (A) 259.193614 (M)	34138-58-4	4.32	7.90
4-methyl-α-pyrrolidinobutirophenone	MPBP[4]	C15H21NO		231.33334 (A) 231.162314 (M)	1214-15-9	3.43	7.70
ARYLCYCLOHEXYLAMINE							
4-metoxi-phenciclidine	4-MeO-PCP[7]	C18H27NO		273.4131 (A) 273.209264491 (M)	2201-35-6	4.33	10.10
Ketamine	KET[7]	C13H16ClNO		237.72524 (A) 237.092042 (M)	6740-88-1	3.35	7.45
	KET-d4	C13H12D4ClNO					
COCAININCS							
Cocaine	COC[8]	C17H21NO4		303.35294 (A) 303.147058 (M)	50-36-2	2.30	8.61
	COC-d3	C17H18D3NO4					
Benzoylecgonine	BECG[9]	C16H19NO4		289.3264 (A) 289.131408101 (M)	519-09-5	9.54	1.71
	BECG-d3	C16H16D3NO4					
Cocaethylene	COCET[10]	C18H23NO4		317.3795 (A) 317.162708229 (M)	529-38-4	2.64	8.77
	COCET-d3	C18H20D3NO4					

Ecgonine methyl ester	ECME[11]	C10H17NO3		199.2469 (A) 199.120843415 (M)	7143-09-1	0.14	9.04
	ECME-d3	C10H14D3NO3					
OPIOIDS							
6-monoacetylmorphine	6- MAM[12]	C19H21NO4		327.3743 (A) 327.147058165 (M)	2784-73-8	1.13	9.08
	6- MAM-d3	C19H18 D3NO4					
Codeine	COD13	C18H21NO3		299.3642 (A) 299.152143543 (M)	76-57-3	1.19	8.21
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine	EDDP[13]	C20H23N		277.4033 (A) 277.183049741 (M)	30223-73-5	4.63	9.64
	EDDP-d3	C20H21D3N					
Heroin	HER[14]	C21H23NO5		369.4110 (A) 369.157622851 (M)	561-27-3	1.58	7.95
	HER-d9	C21H14D9NO5					
Methadone	MET[15]	C21H27NO		309.4452 (A) 309.209264491 (M)	76-99-3	3.93	8.94
	MET-d3	C21H24D3NO					
Morphine	MOR[16]	C17H19NO3		285.3377 (A) 285.136493479 (M)	57-27-2	0.89	8.21
	MOR-d3	C17H16D3NO3					

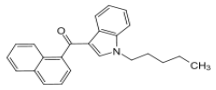
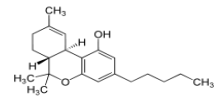
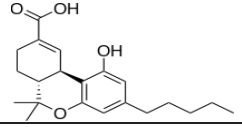
CANNABINOIDS							
JWH-018	JWH-018[17]	C24H23NO		341.44552 (A) 341.177964 (M)	209414-07-3	6.51	---
Tetrahydrocannabinol	THC[17]	C21H30O2		314.4617 (A) 314.224580204 (M)	1972-08-3	5.65	10.6
	THC-d3	C21H27D3O2					
11-Nor-9-carboxy-Δ9-tetrahydrocannabinol	THC-COOH[18]	C21H28O4		344.44462 (A) 344.198759 (M)	104874-50-2	5.14	9.3
	THC-COOH-d3	C21H25D3O4					

Table S8.2. Linearity equation (concentration range LOQ-1000 ng L^{-1}).

Name	Linearity	
	Equation	R^2
AMPHETAMINES		
AMP	y = 0.85818 x + 0.01832	0.9999
MAMP	y = 1.47634 x – 0.01857	0.9945
ETAMINE	y = 0.83805 x – 0.01812	0.9907
EPH	y = 3.06597 x + 0.03240	0.9993
EPHED	y = 0.16889 x + 0.00531	0.9999
MEPHEN	y = 0.28144 x – 0.00661	0.9988
MEP	y = 1.31861 x – 0.02280	0.9976
METONE	y = 0.93323 x + 0.00113	0.9999
MDMA	y = 0.69916 x – 0.01037	0.9933
MDA	y = 256.98135 x – 1.82675	0.9974
MDEA	y = 1.09250 x – 0.01545	0.9918
MBDB	y = 1.09250 x – 0.01545	0.9918
bk-MMBDB	y = 4.95460 x – 0.05574	0.9938
2C-B	y = 0.63545 x – 0.02863	0.9950
NAPH	y = 10.08148 x – 0.05688	0.9977
MDPV	y = 3.22129 x – 0.05061	0.9936
PMA	y = 0.51958 x – 0.01623	0.9956
4-AcO-DIPT	y = 0.00409 x – 1.85813e-4	0.9835
BUF	y = 0.33286 x – 0.00528	0.9954
PIPERAZINES		
mCP	y = 1.59831 x – 0.01850	0.9995
TFMPP[4]	y = 5.19451 x – 0.10160	0.9995
PYRROLIDINOPHENONE		
PPP	y = 2.11916 x – 0.002319	0.9915
alpha-PVP	y = 12.81086 x – 0.16427	0.9951
MDPPP	y = 4.27019 x – 0.00592	0.9933
4-MePPP	y = 5.74971 x – 0.04318	0.9938
4'-MePHP	y = 11.22145 x – 0.32871	0.9934
MPBP	y = 12.61823 x – 0.13288	0.9958
ARYLCYCLOHEXYLAMINE		
4-MeO-PCP	y = 0.44878 x – 0.04646	0.9908
KET	y = 1.43316 x – 0.02540	0.9919
COCAININCS		
COC	y = 0.58410 x – 0.00984	0.9991
BECG	y = 1.21899 x – 0.9938	0.9991
COCET	y = 12.98464 x + 0.12547	0.9994
ECME	y = 2.84568 x – 0.10090	0.9999
OPIOIDS		
6- MAM	y = 1.64494 x – 0.00252	0.9998
COD	y = 1.60488 x – 0.00716	0.9942
EDDP	y = 1.02707 x – 0.00334	0.9999
HER	y = 0.00221 x + 0.00191	0.9886
MET	y = 1.02899 x + 0.00545	0.9992
MOR	y = 0.77705 x – 0.01001	0.9976
CANNABINOIDS		
JWH-018	y = 4.91970 x – 0.00694	0.9999
THC	y = 0.38784 x + 0.04728	0.9997
THC-COOH	y = 0.90973 x – 0.00853	0.9942

Table S8.3. UHPLC-QqQ-MS/MS conditions for all analytes and internal standards.

	T_r (min)	MRM_1 transition (Quantifier)	CE (V)	$MSRM_2$ transition (Qualifier)	CE (V)
AMPHETAMINES					
AMP	2.83	136>91	13	136>65	41
AMP-d_5	2.83	141>93	13	141>92	13
MAMP	3.43	150>119	5	150>91	17
MAMP-d_5	3.43	155>121	9	155>92	17
ETAMINE	5.38	164>119	9	164>91	17
EPH	4.68	166>149	5	166>91	33
EPHED	1.83	164>146	10	164>131	18
MEPHEN	13.07	234>84	17	234>56	53
MEP	6.26	178>160	9	178>145	21
METONE	10.89	208>135	29	208>77	49
METONE-d_3	10.89	211>163	13	211>135	29
MDMA	4.45	194>163	10	194>77	50
MDMA-d_5	4.45	199>165	9		
MDA	3.70	180>163	5	180>105	21
MDA-d_5	3.70	185>85	13	185>65	37
MDEA	10.89	208>105	29	208>77	49
MDEA-d_5	10.89	213>135	21	213>105	33
MBDB	10.89	208>135	17	208>51	77
bk-MMBDB	7.55	236>161	18	236>86	26
2C-B	13.53	260>243	5	260>228	21
NAPH	15.52	282>141	25	282>77	85
MDPV	13.48	276>135	25	276>126	25
PMA	2.03	166>148	9	166>121	21
TRYPTAMINES					
4-AcO-DIPT	13.50	303>202	14	303>114	14
BUF	1.20	205>160	14	205>58	10
PIPERAZINES					
mCPP	8.86	197>154	25	197>91	61
TFMPP	12.46	231>188	21	231>44	21
PYRROLIDINOPHENONES					
PPP	4.12	204>133	14	204>105	30
alpha-PVP	12.98	232>91	22	232>77	58
MDPPP	4.39	248>98	26	248>91	46
4-MePPP	10.78	218>147	14	218>119	14
4'-MePHP	15.25	260>105	18	260>91	50
MPBP	9.98	232>161	14	232>91	34
ARYLCYCLOHEXYLAMINE					
4-MeO-PCP	14.83	274>189	4	274>121	26
KET	11.91	238>125	25	238>89	69
KET-d_4	11.91	242>129	25	242>92	73
COCAINICS					
COC	13.20	304>182	17	304>82	29
COC-d_3	13.20	307>185	17	307>85	53
BECG	12.64	290>168	17	290>105	29
BECG-d_3	12.64	293>171	17	293>105	33
COCET	14.17	318>196	17	318>82	29
COCET-d_3	14.17	321>199	17	321>85	25
ECME	0.73	200>182	17	200>82	25
ECME-d_3	0.73	203>185	17	203>85	29
OPIOIDS					

6- MAM	5.20	328>165	41	328>152	81
6- MAM-d_3	5.20	331>165	30	334>165	41
COD	2.22	300>152	78	300>115	90
EDDP	15.11	279>250	20	279>235	20
EDDP-d_3	15.11	282>235	29	282>115	89
HER	13.11	370>165	53	370>58	25
HER-d_9	13.06	379>166	53	379>61	33
MET	15.84	310>265	9	310>105	25
MET-d_3	15.84	313>268	9	313>77	61
MOR	1.04	286>165	45	286>152	69
MOR-d_3	1.04	289>165	45	289>152	73
CANNABINOIDS					
JWH-018	18.65	342>155	26	342>127	58
THC	19.88	315>193	30	315>123	30
THC-d_3	19.88	318>196	30	318>123	30
THC-COOH	18.81	345>327	9	345>41	73
THC-COOH-d_3	18.81	348>196	33		

Table S8.4. LOD and LOQs, recoveries (100 ng/ L) and matrix effects (n=5) of the whole method.

	LOD[d] (ng/L)	LOQ[d] (ng/L)	River water	
			R (%)	ME (%)
AMPHETAMINES				
AMP	4	12	88 (19)	-9.3
MAMP	2	6	82 (14)	20.1
ETAMINE	4	12	86 (18)	-18.9
EPH	1	3	75 (16)	-5.3
EPHED	10	30	62 (19)	-64.5
MEPHEN	60	120	90 (15)	-38.2
MEP	20	60	75 (17)	-58.2
METONE	20	60	85 (16)	-55.4
MDMA	0.5	1.5	96 (13)	-53.0
MDA	30	90	80 (15)	-0.9
MDEA	20	60	79 (16)	-3.9
MBDB	20	60	79 (17)	-63.6
bk-MMBDB	20	60	86 (18)	-61.9
2C-B	30	90	73 (19)	-13.2
NAPH	4	12	56 (17)	-48.6
MDPV	20	60	61 (18)	67.1
PMA	1	3	89 (12)	-68.5
TRYPTAMINES				
4-AcO-DIPT	60	180	65 (19)	-56.6
BUF	2	6	76 (20)	-67.3
PIPERAZINES				
mCPP	20	60	64 (19)	-77.0
TFMPP	20	60	65 (18)	67.9
PYRROLIDINOPHENONES				
PPP	10	30	75 (16)	-72.2
alpha-PVP	3	9	78 (17)	-63.8
MDPPP	10	30	79 (17)	-65.5
4-MePPP	40	120	74 (15)	-62.5
4'-MePHP	10	30	82 (16)	-58.7
MPBP	3	9	77 (14)	-94.9
ARYLCYCLOHEXYLAMINE				

4-MeO-PCP	2	6	70 (11)	-29.5
KET	10	30	92 (12)	-6.0
COCAINICS				
COC	4	12	101 (19)	-9.2
BECG	0.5	1.5	105 (20)	5.1
COCET	4	12	82 (18)	-11.9
ECME	3	9	62 (15)	13.7
OPIOIDS				
6- MAM	20	60	73 (19)	-6.1
COD	5	15	89 (21)	-18.0
EDDP	20	60	69 (19)	14.0
HER	40	120	70 (20)	-83.7
MET	0.5	1.5	101 (21)	8.3
MOR	4	12	96 (15)	31.9
CANNABINOIDS				
JWH-018	2	6	81 (11)	-0.8
THC	40	120	69 (15)	25.3
THC-COOH	40	120	79 (17)	-4.3

Table S8.5. Detailed concentrations (ng/L) of positive drugs in all sampling points in 2012 and 2013 campaings.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24	P25	P26	P27	P28	P29	P30	P31	P32	P33	P34	P35
2012																																			
MDMA	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	22.77	n.d.	n.d.	n.d.	n.d.	n.d.	n.a	n.d.	n.d.	n.a	n.a	n.a	n.a	n.a	n.a
PMA	n.a.	n.d.	n.d.	n.a.	5.11	n.d.	n.a.	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.a.	18.81	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	19.25	6.18	n.a	n.d.	n.d.	n.a	n.a	n.a	n.a	n.a	n.a
BUF	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	66.77	n.d.	n.d.	n.d.	11.56	n.d.	n.d.	n.d.	n.d.	n.a	9.67	n.d.	n.a	n.a	n.a	n.a	n.a	n.a
4-MeO-PCP	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	37.61	n.d.	n.d.	n.d.	n.d.	n.d.	n.a	n.d.	n.d.	n.a	n.a	n.a	n.a	n.a	n.a
BECG	n.a.	3.26	n.d.	n.a.	n.d.	12.67	n.a.	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	5.82	n.a.	n.d.	23.40	76.76	n.d.	2.91	n.d.	71.07	n.d.	n.d.	n.d.	n.d.	n.a	29.68	3.48	n.a	n.a	n.a	n.a	n.a	n.a
MET	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.a.	n.a.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.29	n.d.	2.02	n.d.	n.d.	n.a	n.d.	39.29	n.a	n.a	n.a	n.a	n.a	n.a
2013																																			
MDMA	n.d.	n.d.	n.d.	n.d.	n.d.	2.39	2.34	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a	n.a	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.21	n.d.	6.05	5.37
EPH	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a	n.a	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	17.65	n.d.	n.d.	11.87	5.28
4-MeO-PCP	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.55	n.a.	n.a	n.a	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
BECG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a	n.a	n.a	5.53	n.d.	n.d.	n.d.	n.d.	n.d.	1.83	n.d.	5.14	3.05	n.d.	4.40	5.51	8.42	12.75
ECME	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	15.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a	n.a	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
COD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a	n.a	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	101.02	n.d.	91.38	81.52
MET	n.d.	40.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.05	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.a.	n.a	n.a	n.a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.29	n.d.	n.d.	10.54	2.59	12.25	8.14

Table S8.6. Values of physico-chemical parameters of the Turia River and rainfall events of each sampling point in 2012 and 2013 campaigns.

Sampling site	Temperature (ºC)		pH		mV		Cond (ds/m)		TDS (ppm)		Res (Ω)		DO (mg/L)		Rainfall (L/m^2) Sampling day		Rainfall (L/m^2) 15 days accumulated		Rainfall (L/m^2) 15 days average	
	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2013	2013	2012	2013	2012	2013	2012	2013	2012	2013
P1	n.a.	18.9	n.a.	8.55	n.a.	-85.8	n.a.	0.48	n.a.	412.3	n.a.	1208	n.a.	9.38	n.a.	0.0	n.a.	9.2	n.a.	0.61
P2	11.1	18.1	7.93	8.63	-47.8	-95.1	0.68	0.48	366.3	383.8	1365	1293	8.56	9.57	0.0	0.0	68.8	9.2	4.59	0.61
P3	10.5	--	8.15	--	-62.0	--	0.80	--	414.1	--	1204	--	9.65	--	0.0	0.0	68.8	9.2	4.59	0.61
P4	n.a.	19.6	n.a.	8.49	n.a.	-80.3	n.a.	0.64	n.a.	555.8	n.a.	899.7	n.a.	10.46	n.a.	7.0	n.a.	3.0	n.a.	0.20
P5	14.4	15.6	8.23	8.67	-62.9	-99.5	0.68	1.19	438.3	843.3	1141	592.7	6.72	9.73	0.0	2.8	54.4	10.0	3.63	0.67
P6	13.7	17.1	8.25	8.76	-59.7	-82.2	1.11	0.87	693.9	650.3	726.8	767.3	8.43	9.23	0.0	2.8	54.4	10.0	3.63	0.67
P7	n.a.	17.6	n.a.	9.04	n.a.	-90.0	n.a.	0.87	n.a.	674.5	n.a.	741.5	n.a.	9.47	n.a.	2.8	n.a.	10.0	n.a.	0.67
P8	n.a.	14.8	n.a.	9.06	n.a.	-117	n.a.	0.17	n.a.	124.8	n.a.	3973	n.a.	10.08	n.a.	7.8	n.a.	49.0	n.a.	3.27
P9	11.7	13.5	8.20	9.06	-59.1	-96.7	0.72	0.65	396.1	401.6	1259	1240	7.94	11.73	0.0	7.8	54.0	49.0	3.60	3.27
P10	11.2	12.8	8.15	9.14	-61.5	-99.1	0.71	0.70	383.3	415.0	1332	1200	9.03	12.10	0.0	7.8	54.0	49.0	3.60	3.27
P11	n.a.	14.3	n.a.	8.92	n.a.	-88.5	n.a.	0.69	n.a.	463.3	n.a.	1074	n.a.	11.36	n.a.	7.8	n.a.	49.0	n.a.	3.27
P12	12.5	16.3	8.45	9.07	-71.2	-88.2	0.74	0.70	438.6	515.4	1133	964.7	10.02	11.45	0.0	1.4	56.7	17.4	3.78	1.16
P13	12.5	17.2	8.30	8.66	-69.1	-94.5	0.77	0.68	449.2	539.5	1113	920.2	8.40	10.16	0.0	1.4	56.7	17.4	3.78	1.16
P14	12.3	14.0	8.40	9.09	-66.2	-116.4	0.95	0.60	548.4	397.3	915.4	1255	9.22	12.78	0.02	0.0	53.2	32.0	3.55	2.13
P15	n.a.	--	n.a.	--	n.a.	--	n.a.	--	n.a.	--	n.a.	--	n.a.	--	n.a.	0.0	n.a.	32.0	n.a.	2.13
P16	12.6	15.3	8.28	8.91	-63.2	-109.2	1.38	1.10	889.0	772.0	617.1	644.6	8.19	10.33	0.0	2.8	54.4	10.0	3.63	0.67
P17	13.4	n.a.	8.35	n.a.	-77.1	n.a	0.96	n.a	577.8	n.a	864.7	n.a	10.22	n.a	0.0	n.a	57.8	n.a	3.85	n.a
P18	13.1	n.a.	8.50	n.a.	-76.5	n.a	0.96	n.a	576.4	n.a	864.9	n.a	10.21	n.a	0.0	n.a	57.8	n.a	3.85	n.a
P19	18.2	n.a.	8.45	n.a.	-63.4	n.a	1.22	n.a	960.1	n.a	522.4	n.a	7.03	n.a	0.0	n.a	92.6	n.a	6.17	n.a
P20	19.3	n.a.	8.16	n.a.	-65.1	n.a	1.25	n.a	1045	n.a	480.2	n.a	6.28	n.a	9.80	n.a	92.6	n.a	6.17	n.a
P21	15.0	19.1	8.26	8.09	-63.8	-64.3	0.98	0.77	658.8	650.2	775.2	766.3	10.13	7.81	0.0	1.2	7.2	0.0	0.48	0.0
P22	14.0	18.6	7.89	7.50	-51.6	-29.6	0.82	0.77	520.7	631.5	956.5	789.9	9.51	7.30	0.0	1.2	7.2	0.0	0.48	0.0
P23	18.8	17.9	8.26	8.06	-60.8	-61.4	1.13	1.06	910.8	859.8	548.3	578.7	7.94	9.15	0.0	0.6	9.4	0.0	0.63	0.0
P24	18.9	19.2	8.36	8.64	-74.4	-93.4	1.14	1.05	921.7	907.7	541.8	545.6	8.16	9.11	0.0	0.6	9.4	0.0	0.63	0.0
P25	18.2	21.1	8.29	8.34	-69.0	-78.8	1.14	1.06	891.9	984.0	560.4	506.2	8.90	9.51	0.0	0.6	12.0	0.0	0.80	0.0
P26	20.2	21.4	8.75	8.11	-82.9	-68.2	1.13	1.05	954.9	958.6	522.2	521.0	9.08	9.51	0.0	0.6	12.0	0.0	0.80	0.0
P27	n.a.	22.2	n.a.	8.71	n.a.	-99.4	n.a.	1.14	n.a.	1117	n.a.	447.2	n.a.	11.45	n.a.	0.0	n.a.	7.8	n.a.	0.52
P28	16.5	21.0	8.24	8.69	-62.1	-86.9	1.22	1.14	906.1	1056	536.0	472.6	8.38	11.68	0.0	0.0	0.2	7.0	0.07	0.47
P29	12.0	21.9	8.39	8.55	-72.7	-84.0	1.34	1.15	750.0	1152	666.0	434.0	7.30	7.64	4.2	0.0	35.0	7.0	2.33	0.47
P30	n.a.	22.7	n.a.	8.56	n.a.	-102.5	n.a.	1.13	n.a.	1098	n.a.	455.4	n.a.	10.60	n.a.	0.0	n.a.	5.4	n.a.	0.36
P31	n.a.	22.9	n.a.	8.44	n.a.	-85.0	n.a.	1.11	n.a.	1116	n.a.	443.6	n.a.	9.93	n.a.	0.0	n.a.	5.4	n.a.	0.36
P32	n.a.	24.2	n.a.	8.84	n.a.	-103.5	n.a.	1.21	n.a.	1286	n.a.	389.0	n.a.	10.17	n.a.	0.0	n.a.	4.4	n.a.	0.29
P33	n.a.	28.0	n.a.	8.17	n.a.	-72.2	n.a.	1.28	n.a.	1589	n.a.	313.0	n.a.	7.31	n.a.	0.0	n.a.	2.2	n.a.	0.15
P34	n.a.	30.0	n.a.	8.59	n.a.	-89.3	n.a.	1.68	n.a.	2291	n.a.	218.2	n.a.	5.14	n.a.	0.0	n.a.	2.2	n.a.	0.15
P35	n.a.	29.0	n.a.	8.59	n.a.	-96.7	n.a.	1.40	n.a.	1817	n.a.	274.6	n.a.	7.22	n.a.	0.0	n.a.	4.4	n.a.	0.29

Table S8.7. Satatistically significant Pearson correlations between the studied drugs of abuse in surface water

	EPH	MDMA	PMA	BUF	4-MeO-PCP	BECG	ECME	COD
EPH								
MDMA	0.334*							
PMA								
BUF								
4-MeO-PCP		0.573**						
BECG				0.688**				
ECME								
COD	0.547**	0.662**				0.289**		
MET	0.271*	0.311*						0.535**

** Significant correlation at 0.01 level.
* Significant correlation at 0.05 level.

Table S8.8. Satatistically significant Pearson correlations between the studied drugs of abuse and qualiy parameters and rainfall in surface water

	pH	Temperature (ºC)	Cond (dS/m)	TDS (ppm)	Res (Ω)	DO (mg/L)	Rainfall sampling day (L/m^2)	Rainfall 15 days accumulated (L/m^2)	Rainfall 15 days average (L/m^2)
EPH		0.439**	0.277*	0.421**	-0.424**	-0.302*			-0.333*
MDMA		0.305*		0.319*	-0.316*				-0.352**
PMA									
BUF									
4-MeO-PCP									
BECG			0.294*						
ECME								-0.317*	
COD		0.482**	0.310*	0.469**	-0.470**	-0.319*			-0.357**
MET						-0.278*			

** Significant correlation at 0.01 level.
* Significant correlation at 0.05 level.

Table S8.9. Multiple stepwise regression between the different drugs of abuse.

Y	B_0	*Bj*	*Xj*	R^2	*Sig.**
EPH	0.025	B_1=0.290	X_1= [COD]	0.299	0.000
MDMA	0.010	B_1= 0.398 B_2= 0.650	X_1= [COD] X_2= [4-MeO-PCP]	0.803	0.000
4-MeO-PCP	0.001	B_1= 0.533	X_1= [MDMA]	0.328	0.000
BECG	0.213	B_1= 1.111	X_1= [BUF]	0.474	0.000
COD	-0.008	B_1= 0.934 B_2= 0.692	X_1= [MDMA] X_2= [EPH]	0.577	0.001
MET	0.110	B_1= 0.458	X_1= [COD]	0.287	0.000

***** Sig.: Significance $p<0.05$.

Table S8.10. Multiple stepwise regression between the different drugs of abuse and quality parameters and rainfall in surface waters.

Y	B_0	*Bj*	*Xj*	R^2	*Sig.**
EPH	-1.086	B_1= – 0.937	X_1= [T]	0.192	0.001
MDMA	0.095	B_1= -0.196	X_1= [Rainfall_Aver$_{15}$]	0.124	0.010
BECG	0.340	B_1= 0.941	X_1= [Cond]	0.086	0.033
COD	-2.256	B_1= 1.941	X_1= [T]	0.232	0.000
MET	1.487	B_1= -1.378	X_1= [DO]	0.077	0.044
ECME	0.003	B_1= 0.151	X_1= [Rainfall_Samp_day]	0.100	0.021

***** Sig.: Significance $p<0.05$.
T: temperatura
Aver$_{15}$: rainfall average during 15 days before sampling day
Sampl_day: rainfall at sampling day

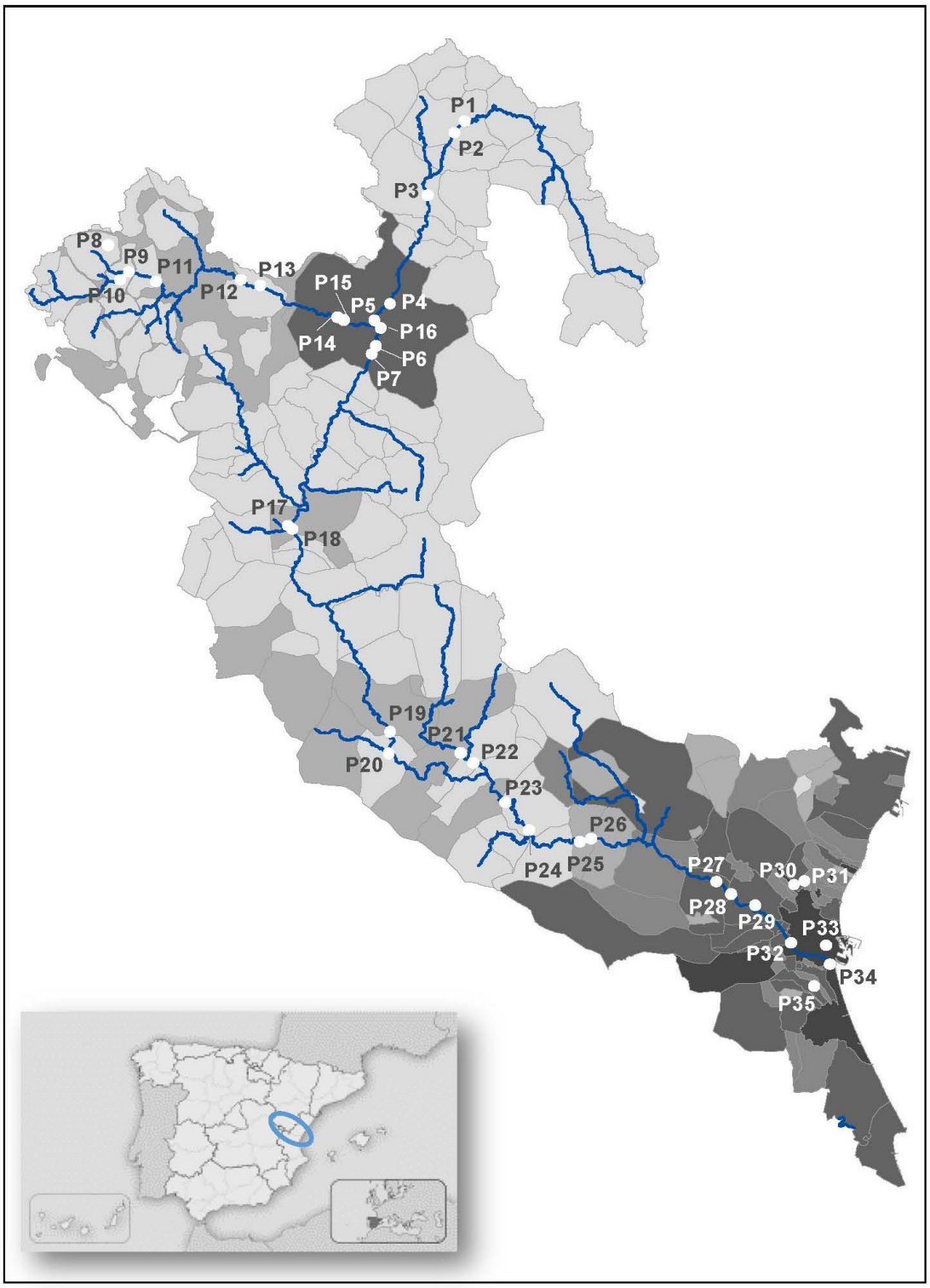


Figure S8.1. Location of sampling points in Turia River basin.

PUBLICACIÓN CIENTÍFICA 9

Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance

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Water Research (enviada)

Enantioselective transformation of fluoxetine in water and its ecotoxicological relevance

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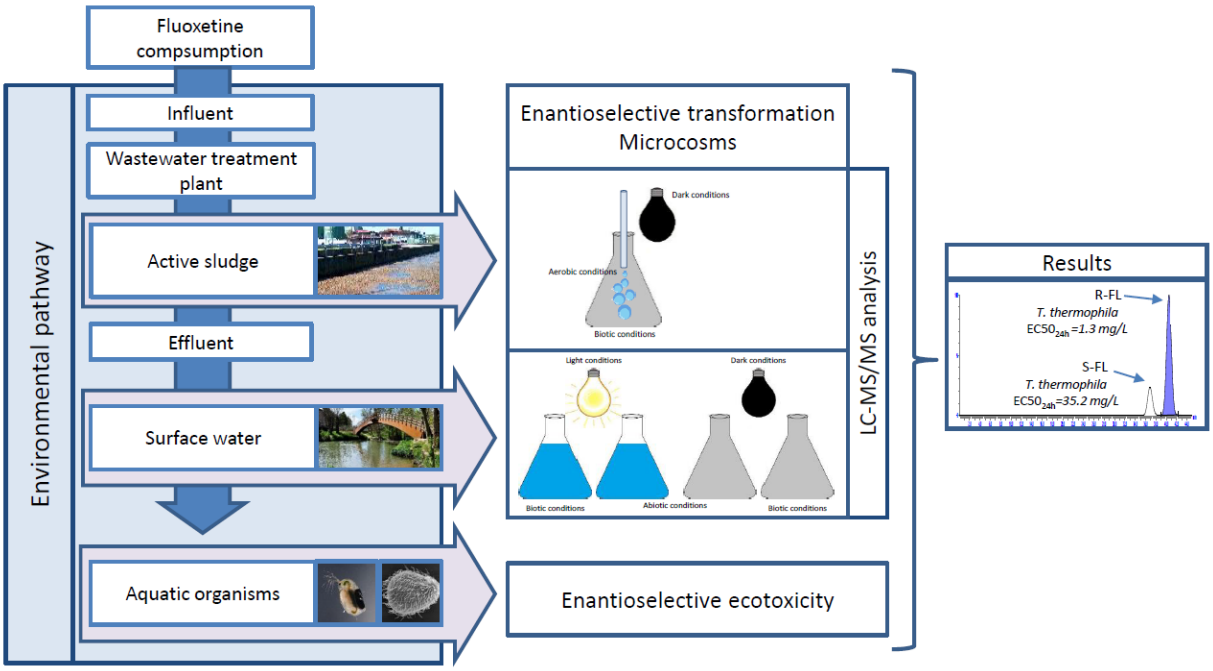
Highlights

- *(S)*-fluoxetine (FL) undergoes preferential degradation during activated sludge treatment
- (*S*)-norfluoxetine formation is favored to (R)-norfluoxetine
- (*R*)-FL is 30 times more toxic to T. thermophila than (*S*)-FL
- Despite the decrease in FL concentration, accumulation of (*R*)-FL leads to higher toxic effects

Graphical abstract



Abstract

The research reported in this manuscript is aimed at testing the hypothesis that degradation of fluoxetine (FL), formation of its main metabolite norfluoxetine (NFL) and their biological effects in the aqueous environment are stereoselective and biological in nature. River simulating microcosms revealed that degradation of FL takes place via photochemical and mildly-enantioselective processes favoring the (*R*)-enantiomer. However, a pronounced enantioselectivity favoring *(S)*-FL (leading to the formation of (*S*)-NFL) was observed during activated sludge simulating microcosms. This is in contrast to metabolic processes in humans, which favor the (*R*)-enantiomer. Toxicity tests showed that while there is no significant enantioselectivity in the toxic response from *Daphnia magna* to both FL and NFL, a strong enantiomer-specific toxicity is observed in the case of *Tetrahymena thermophila* ((*R*)-FL is 30× more toxic than (*S*)-FL), protozoa that are utilized during activated sludge treatment. Therefore, preferential degradation of (*S*)-FL in activated sludge microcosms is not surprising. Unfortunately, this also indicates that FL gets enriched with more toxic (*R*)-FL. This accumulation of (*R*)-FL is likely to have detrimental effects on protozoa. One can assume that, if stereochemistry of FL is not considered, a decreased concentration of FL as a result of activated sludge treatment leads to decreased biological impact. Such an approach (as currently applied in ERA) can lead to incorrect conclusions and detrimental impacts on environmental health. Our study proves that despite the overall decrease in FL concentration, accumulation of toxic (*R*)-FL and formation of toxic (*S*)-NFL in activated sludge leads to higher than estimated toxicological effects.

Keywords: fluoxetine, chiral drugs, enantioselective degradation, ecotoxicity

1. Introduction

Fluoxetine (FL) is a diphenhydramine derivative and selective serotonin reuptake inhibitor (SSRI). It is used to treat a variety of mental health problems such as depression, panic, anxiety, or obsessive-compulsive symptoms. There was a 165% increase in the prescribing of antidepressant drugs in England between 1998 and 2012 (an average of 7.2% per year) (Spence et al. 2014). Indeed, FL is the fourth most prescribed antidepressant in England, and accounts for 11.3% of all antidepressant drug use (HSCIC 2015).

FL is extensively metabolized to norfluoxetine (NFL) and several other metabolites such as FL glucuronide, NFL glucuronide, *para*-trifluoromethylphenol and hippuric acid. The principal metabolite, NFL, is formed by *N*-demethylation of FL. The potency and selectivity of NFL's SSRI activity is similar to that of the parent drug. The elimination of FL accounts for 80% excreted in the urine (as 11.6% FL, 7.4% FL glucuronide, 6.8% NFL, 8.2% NFL glucuronide, >20% hippuric acid, 46% other) and approximately 15% excreted in the feces (NCBI).

Recent research studies have shown that most pharmaceuticals, including FL and NFL, enter the aquatic environment via (un)treated communal wastewater. Both FL and NFL have been detected in wastewater and receiving waters at levels ranging from ng L^{-1} to μg L^{-1} (Vasskog et al. 2008, Fernandez et al. 2010, Bagnall et al. 2012b, Lazzara et al. 2012, Guedes-Alonso et al. 2013, Ribeiro et al. 2014, Silva, Santos et al. 2014, López-Serna et al. 2013, Silva, Pereira et al. 2014, Birch et al. 2015, Evans et al. 2015b). Furthermore, they were found in the tissue of fish collected near municipal wastewater discharges. Both FL and NFL remain biochemically active in the environment and can have marked effects on the morphology, physiology, and behavior

of different species (Ramirez et al. 2007, Ramirez et al. 2009, Smith et al. 2010, Fernandez et al. 2010, Silva, Pereira et al. 2014, Silva et al. 2015).

Despite extensive research on fate and effects of FL, there has been very little attention paid to the stereochemistry of FL and its possible environmental impacts. FL has one chiral carbon in its structure and as a result it exists in two enantiomeric forms as (*S*)-FL and (*R*)-FL. Similarly, NFL exists in two enantiomeric forms as (*S*)-NFL and (*R*)-NFL. Enantiomers of the same drug have identical physicochemical properties but may differ in their biological properties. Thus, chiral drugs can undergo stereoselective mechanisms controlling their fate such as distribution, metabolism and excretion, as these processes (due to stereoselective interactions of enantiomers with biological systems) usually favor one enantiomer over the other. This leads to process-dependent changes in the enantiomeric composition of chiral compounds (Bagnall et al. 2012b). Metabolism of FL was found to be enantioselective in humans, with the (*R*)-enantiomer being metabolised faster than (*S*)-enantiomer (Caccia, 1998). Additionally, due to different pharmacological activity, enantiomers of chiral drugs can differ in their biological actions, potency and toxicity (Kasprzyk-Hordern, Kondakal et al. 2010). Enantiomers of FL have similar potency as inhibitors of the serotonin reuptake pump whereas enantiomers of NFL act differently, with (*S*)-NFL showing higher inhibition capacity (Fuller et al. 1992).

Ecotoxicity of FL (and other pharmaceuticals) is currently assessed for the racemate, as FL is marketed as a racemic mixture of two enantiomers. ERA (environmental risk assessment) approaches need to be re-evaluated as they are based on a simplistic assumption that FL present in the environment is racemic (Bagnall et al. 2012b). Indeed, limited research indicates that FL and NFL are present in the aqueous environment as non-racemic mixtures, i.e. enriched with one enantiomer (MacLeod al.

2007, Bagnall et al. 2012a, Barclay et al. 2012b, Kasprzyk-Hordern and Baker 2012, Ribeiro et al. 2014, Ma et al. 2016). Furthermore, FL was found to undergo enantioselective transformation during wastewater treatment (Ribeiro et al. 2014, Kasprzyk-Hordern, 2010). In a recent study, Barclay et al. (2011) found a slight enrichment of FL and NFL with (*S*)-enantiomer in both raw and treated wastewater (Barclay et al. 2012b). In contrast, MacLeod et al. (2007) reported that in their monitoring study FL was enriched with (*R*)-enantiomer in wastewater with preferential degradation of this enantiomer during wastewater treatment leading to enrichment of FL with (*S*)-enantiomer (MacLeod et al. 2007, Ribeiro et al. 2014).

FL is often used as a model compound for assessing SSRI impact on aquatic organisms such as zebrafish, Japanese medaka, goldfish, gulf toadfish, rainbow trout, fathead minnows and polychaete worms (*Capitella teleta)* (Foran et al. 2004, Stanley et al. 2007, Lister et al. 2009, Mennigen et al. 2009, Morando et al. 2009, Grabicova et al. 2014, Méndez and Barata 2015,). FL was reported as toxic at low concentrations to several aquatic species (Brooks et al. 2003, Foran et al. 2004, De Andrés et al. 2009, Gonzalez-Rey and Bebianno 2013). In fact, FL and NFL were proposed as being among 10 pharmaceuticals potentially dangerous for the environment (Ribeiro et al. 2014). Limited studies have tested the enantioselective toxicity of FL and NFL. These include work by Stanley et al. (2007) and de Andrés (2009) (Stanley et al. 2007, De Andrés et al. 2009). Enantioselective toxicity of FL was also demonstrated for *Primephales promelas* and *Tetrahymena thermophila*, where (*S*)-FL was found to be more toxic than its respective enantiomer (Stanley et al. 2007, De Andrés et al. 2009). On the other hand (*R*)-FL was considered more harmful for *Pseudokirchneriella subcapitata* (De Andrés et al. 2009). NFL was reported to be more active than the parent compound (Stokes and Holtz 1997). Furthermore, a few studies have established the toxicology of NFL. One of

them determined the enantiomers of NFL have markedly different potencies as inhibitors of the uptake of serotonin with (*S*)-NFL being more potent than the (*R*)-enantiomer in rats (Fuller et al. 1992).

The above discussion clearly indicates that current ERA approaches that do not recognise stereochemistry of chiral pharmaceuticals are inaccurate and could lead to incorrect conclusions being drawn regarding the ecotoxicological effects of chiral drugs. The limited amount of work in the area of stereochemistry-induced fate and effects of FL (and pharmaceuticals in general) is mainly due to lack of enantioselective analytical methods. Such analytical methods are essential to gather accurate data needed for comprehensive ERA of these compounds (Ribeiro et al. 2014).

The main objectives of this study were:

i) To develop a new, fast and sensitive analytical method for the detection and quantification of enantiomers of FL and NFL utilizing chiral liquid chromatography coupled with tandem mass spectrometry.

ii) To undertake mechanistic study of the degradation of FL and NFL formation in controlled river water and activated sludge simulating microcosm experiments.

iii) To verify enantiomer-specific toxicity of FL and NFL in aquatic species.

This is, to the authors' knowledge, the first report studying degradation of FL in river and activated sludge simulating microcosms including effects of microbial degradation and photolysis. The research reported in this manuscript tests the hypothesis that degradation of chiral compounds is stereoselective and biological in nature.

2. Experimental

2.1. Chemicals and materials

HPLC-grade methanol (MeOH), ethanol (EtOH), ammonium acetate (AAC, 99%), formic acid (FA, 98%) were purchased from Sigma Aldrich (Cambridge, UK). Ultrapure water (HQ water) was supplied by a Milli-Q system (PURELAB, Elga, UK).

The reference standards, *rac*-FL and *rac*-NFL and the internal standard (IS) FL-d_5 were purchased from LGC Standards (Teddington, UK). Enantiomerically pure (*S*)-FL, (*R*)-FL standards were purchased from Sigma-Aldrich. Enantiomerically pure (*S*)-FL, (*R*)-FL, (*S*)-NFL and (*R*)-NFL for toxicity studies were synthesized at the University of Bath (see section: 2.2). All standards and ISs were of the highest purity available (>97%). Structures, molecular formula and molecular weights of target enantiomers are summarized in **Table S9.1** in the supplementary information.

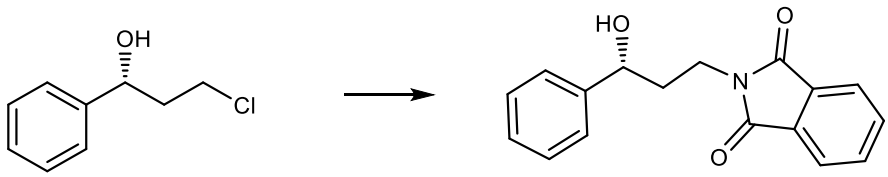
Stock solutions of the individual compounds were purchased in MeOH at a concentration of 1 mg mL^{-1} or 0.1 mg mL^{-1} and stored in the dark at -16°C. Working solutions were prepared by diluting stock solutions in mobile phase or MeOH on a daily basis and stored at 4°C.

All glassware was deactivated with dimethyldichlorosilane (5% DMDCS in toluene, Sigma-Aldrich) to minimize sample loss through adsorption of basic analytes onto –OH sites present on the glass surface (Kasprzyk-Hordern et al. 2007). Oasis HLB (60mg, 3mL, Waters, UK) was used for solid phase extraction (SPE). HQ water (Elga, Marlow, UK), river water (collected in South-West England) and activated sludge (collected from a local wastewater treatment plant) were used for method development and validation.

2.2. Synthesis of FL and NFL enantiomers

This procedure employs 3-chloro-1-phenyl-1-propanol as starting material and source of chirality. This was purchased from Sigma-Aldrich chemical company: (*R*)-enantiomer product #338419; (*S*)-enantiomer product #324612. Both were certified as having 99% enantiomeric excess. Experimental procedures are described for the (*R*)-enantiomer; identical procedures were carried out with the (*S*)-enantiomer of starting material to synthesize (*S*)-NFL and (*S*)-FL.

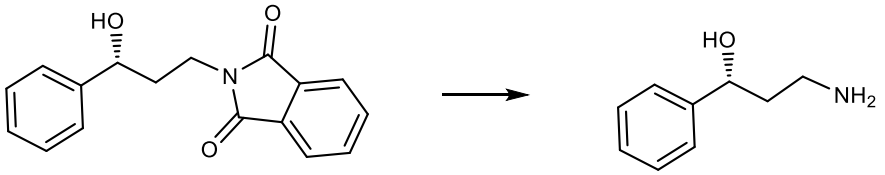
Step 1: (*R*)-3-phthalimido-1-phenylpropanol



At room temperature, to a stirring suspension of potassium phthalimide (3.93 g, 21.25 mmol) in dry dimethylformamide (DMF) (115 mL) was added (*R*)-3-chloro-1-phenyl-1-propanol (3.00 g, 17.65 mmol) in dry DMF (5 mL). The reaction mixture was heated to 90°C and left to stir for 2 hours, until completion was observed by thin layer chromatography (TLC.) To the cooled reaction mixture was added H_2O (300 mL), and extracted with diethyl ether (2 × 300 mL). The combined organic extracts were washed with a saturated solution of LiCl (300 mL), brine (300 mL), dried over $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to give (*R*)-3-phthalimido-1-phenylpropanol as a white powder (4.30g, 88%); mp 78-79°C; R_f 0.36 (3:1 Petrol/ethyl acetate). δ_H (250 MHz, $CDCl_3$) 7.86-7.83 (2H, m, ArH), 7.74-7.71 (2H, m, ArH), 7.36-7.21 (5H, m, ArH), 4.69 (1H, t, *J* 6.5 Hz, CHOH), 3.91 (2H, t, *J* 6.5 Hz, CH_2N), 2.13-

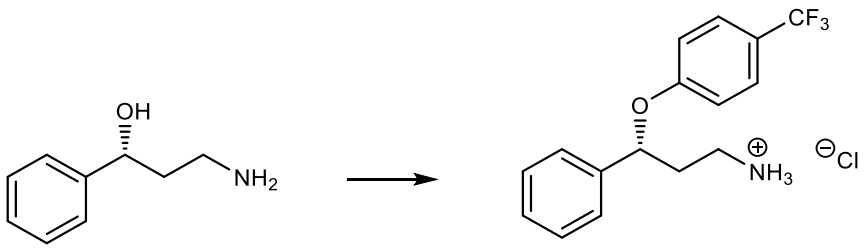
2.05 (2H, m, C<u>H</u>$_2$CHOH); δ_C (300 MHz, $CDCl_3$) 168.8, 143.5, 134.0, 131.9, 128.4, 127.4, 125.6, 123.3, 71.2, 37.6, 34.8 (**Figure S9.1** in the supplementary information)

Step 2: (*R*)-3-amino-1-phenyl-1-propanol



At room temperature, to a stirred solution of (*R*)-3-phthalimido-1-phenylpropanol (4.10 g, 14.5 mmol) in EtOH (90 mL) was added hydrazine hydrate (2.09 mL, 43.5 mmol). The reaction mixture was stirred for 1 hour and then heated to reflux for 2 hours. The reaction mixture became thick and cloudy upon heating, and when cooled, precipitate was filtered off. Recovered filtrate was concentrated under reduced pressure, diluted with dichloromethane (DCM) (10 mL) and filtered, washed with DCM (2 × 5 mL). The recovered filtrate was concentrated *in vacuo* to give the title compound (*R*)-3-amino-1-phenyl-1-propanol as a brown oil (1.85 g, 85%); R_f 0.09 (100:10:1 DCM/MeOH/ Et_3N). δ_H (300 MHz, dimethylsulfoxide (DMSO-d_6)) 7.34-7.27 (4H, m, Ar<u>H</u>), 7.24-7.17 (1H, m, Ar<u>H</u>), 4.66 (1H, dd, *J* 7.0, 6.0 Hz Hz C<u>H</u>OH), 2.71-2.60 (2H, m, C<u>H</u>$_2$N), 1.69-1.62 (2H, m, C<u>H</u>$_2$CHOH); δ_C (75 MHz, DMSO-d_6) 146.6, 128.0, 126.6, 125.7, 71.3, 42.2, 38.9 (**Figure S9.2** in the supplementary information)

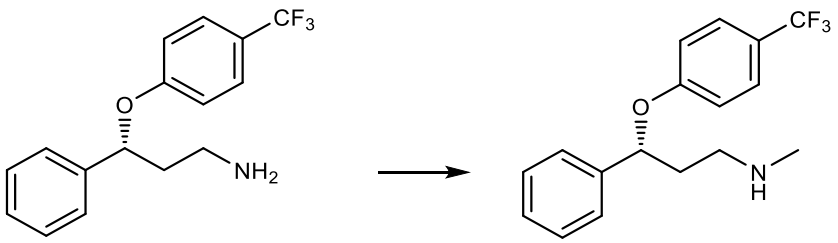
Step 3: (*R*)- 3-Phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine•HCl [(*R*)-NFL hydrochloride salt]



At 0°C, to a stirred suspension of sodium hydride (60% in oil, 0.73 g, 18.34 mmol) in DMSO (3.0 mL) was added (*R*)-3-amino-1-phenyl-1-propanol (1.85 g, 12.23 mmol) in DMSO (1.0 mL). The reaction mixture was stirred at 55°C for 30 min and 4-fluorobenzotrifluoride (3.01 g, 18.34 mmol) in 1.85 mL DMSO was added dropwise. The resulting mixture was heated for 1 hour at 90°C, until completion was observed by TLC. The mixture was cooled to 0°C, and diluted with aqueous 1 N NaOH (20 mL). Toluene was used to extract the product (3 × 20 mL), and combined organic extracts were dried over $MgSO_4$ and filtered. The crude product was purified by column chromatography (100:0:1 to 100:6:1 DCM/MeOH/Et_3N) to give (*R*)-3-phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine as a brown oil (2.10 g, 58%). Product (1.0 g, 3.39 mmol) was dissolved in 4 M HCl in dioxane (10.0 mL, 40 mmol), and left to stir for 2 hours. Reaction mixture was concentrated *in vacuo,* and recrystallized (50 mL of 3:2 diethyl ether/hexane) to give (*R*)-3-Phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine•HCl as a white solid (0.80 g, 71%); mp 128-129 °C; R_f 0.04; δ_H (250 MHz, $CDCl_3$) 8.45 (3H, br s, N<u>H</u>$_3$) 7.39 (2H, d, *J* 8.5 Hz, Ar<u>H</u>), 7.27-7.32 (5H, m, Ar<u>H</u>), 6.91 (2H, d, *J* 8.5 Hz, Ar<u>H</u>), 5.42 (1H, dd, *J* 7.5, 4.5 Hz, C<u>H</u>O), 3.18 (2H, app t, *J* 5.5 Hz, C<u>H</u>$_2$$CH_2$NH), 2.47-2.27 (2H, m, C<u>H</u>$_2$N); δ_C (300 MHz, $CDCl_3$): 159.5, 139.0, 129.1, 128.4, 126.7 (q, $^3J_{CF}$ 3.8 Hz), 125.7, 124.2 (q, $^1J_{CF}$ 270 Hz), 123.3 (q, $^2J_{CF}$ 32.6 Hz), 115.9, 77.4, 36.9, 36.0; ν_{max} (film) 3385 (N-H), 2891 (C-H), 2015, 1613 cm^{-1}; $[\alpha]_D$

+14.0° (*c* 1, $CHCl_3$); for (*S*)-enantiomer: $[\alpha]_D$ –15.0° (*c* 1, $CHCl_3$) (**Figure S9.3** in the supplementary information).

Step 4: (*R*)-*N*-Methyl-3-(4-trifluoromethylphenoxy)-3-phenylpropylamine [(*R*)-FL]



At room temperature, to a stirred solution of 3-phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine ((*R*)-NFL) (1.0 g, 3.38 mmol) and methyl chloroformate (0.29 mL, 3.72 mmol) in DCM (15.0 mL) was added aqueous K_2CO_3 (2.33 g, 16.89 mmol in 30 mL H_2O). The reaction mixture was vigorously stirred for 20 minutes, until completion was observed by TLC, dyed with ninhydrin. The organic phase was separated and the aqueous phase extracted with DCM (2 × 30 mL). The combined organic extracts were dried over $MgSO_4$ and filtered. The filtrate was concentrated *in vacuo* to yield intermediate carbamate as a pale yellow oil. At 0 °C, to a stirring suspension of $LiAlH_4$ (0.25 g, 6.59 mmol) in dry tetrahydrofuran (THF) (15.0 mL) was added dropwise a solution of the intermediate carbamate in dry THF (5.0 mL). The reaction mixture was gradually heated to reflux for 2 hours. To the cooled mixture were cautiously added 0.25 mL of water, followed by 0.25 mL of 2N NaOH, and 0.75 mL of water, in that order. The solution was dried over $MgSO_4$ and filtered. The filtrate was concentrated over reduced pressure, and the crude product was purified by column chromatography (100:0:1 to 100:1:1 EtOAc/MeOH/Et_3N) to give (*R*)-*N*-Methyl-3-(4-

trifluoromethylphenoxy)-3-phenylpropylamine (*R*-FL) as a pale yellow oil (0.84 g, 80%); R_f 0.09 (100:1:1 EtOAc/MeOH/Et_3N); δ_H (300 MHz, $CDCl_3$) 7.43 (2H, d, *J* 8.5 Hz, ArH), 7.34-7.23 (5H, m, ArH), 6.90 (2H, d, *J* 8.5 Hz, ArH), 5.31 (1H, dd, *J* 8.0, 4.5 Hz, CHO), 2.82-2.66 (2H, m, CH_2CH_2NH), 2.44 (3H, s, CH_3), 2.27-2.16 (1H, m, CHHN), 2.08-1.97 (1H, m, CHHN), 1.69 (1H, br. s, NH); δ_C (300 MHz, $CDCl_3$) 160.5, 141.0, 128.8, 127.8, 126.7 (q, $^3J_{CF}$ 3.8 Hz), 125.7, 115.7, 78.5, 48.2, 38.6, 36.4 (signals for -CF_3 and C-CF_3 were not observed); ν_{max} (film) 3033 (ArC-H), 2937 (ArC-H), 2846 (C-H), 2796, 1614 cm^{-1}; $[\alpha]_D$ + 3.0° (*c* 1, $CHCl_3$); for (*S*)-enantiomer: $[\alpha]_D$ – 3.0° (*c* 1, $CHCl_3$) (**Figure S9.4** in the supplementary information).

2.3. Microcosm bioreactors

2.3.1. River water simulating microcosms

River water microcosm experiments were conducted in light (L) and dark (D) conditions (to study photochemical processes) and biotic (B) or abiotic (A) conditions with or without sodium azide (an inhibitor of microbiological processes) respectively as shown in **Figure 9.1**. Four microcosm bioreactors were investigated in duplicate thus eight autoclaved conical flasks were used as bioreactors in microcosm experiments. Each bioreactor was filled with 2 L of river water collected from a local river and spiked with racemic standard of *S*/*R* (±) FL to obtain a final concentration of 1 µg L^{-1}. Abiotic bioreactors were spiked with sodium azide at a concentration of 1 g L^{-1} to inhibit biotic processes. Two replicates of biotic and abiotic bioreactors were exposed to light and another two replicates of each bioreactor were kept in the dark. Light conditions were simulated with an Osram 400 W HQI BT daylight lamp during 8 h each day. Average photon flux measured at the level of the bottle base was 395 µmol m^{-2} s^{-1}. Dark conditions were simulated covering up the flask with foil. Magnetic stirrers were used to ensure good mixing. The experiment was carried out during 16 days. Samples (50

mL each) were collected every day, with the exception of weekends. IS was added to each sample (to obtain a final concentration of 100 ng mL^{-1}). Samples were then frozen to prevent compound degradation until their analysis.

Dissolved oxygen (DO), pH and temperature (T) were analyzed during sampling period and total suspended solids (TSS), NO_2^-, NH_4, and chemical oxygen demand (COD) were analyzed at the beginning of the experiment period (**Table S9.2** in supplementary information).

2.3.2. Activated sludge simulating microcosms

Activated sludge microcosm experiments were conducted in the dark and aerobic conditions. Three microcosm bioreactors were investigated in duplicate, thus six autoclaved conical flasks were used as bioreactors in the microcosm experiments. They were filled with 2 L of fresh activated sludge collected from a local wastewater treatment plant utilizing activated sludge process. One bioreactor remained un-spiked and the other two bioreactors were spiked with a racemic standard of *S*/*R* (±) FL to obtain concentrations of either 10 or 100 μg L^{-1}. Dark conditions were simulated by covering up the flask with foil. Aerobic conditions were obtained using air from BOC air cylinder and a thorough mixing was maintained using magnetic stirrers. The experiment was 24 hours long. Samples (100 mL each) were taken at the following time intervals: 0, 0.5, 1, 1.5, 2, 3, 5, 8, 12 and 24 h. After sample collection, IS was added to each sample to obtain a final concentration of 100 ng mL^{-1}. Collected samples were subsequently frozen until their analysis to prevent degradation of compounds.

DO, pH, T and TOC, were analyzed throughout the experimental period and TSS, NO_3^-, NO_2^-, NH_4 and COD were analyzed at the beginning of the experiment at t = 0 min (**Table S9.3** in supplementary information).

2.3.3. Kinetics – activated sludge simulating microcosms

The compounds studied are characterized as having low volatility and therefore volatilization was not considered as a potential removal pathway in studied microcosms. Photodegradation was also not considered (not relevant) under tested activated sludge conditions. Therefore the two important degradation mechanisms to consider were biodegradation and sorption to sludge. FL was reported to have high sorption affinity towards particulate matter (Baker and Kasprzyk-Hordern 2011). However, in this study, sorption equilibrium was assumed and therefore sorption could be considered negligible. This is because sorption is assumed to be fast when compared to biological degradation (Joss et al. 2006). Section 3.2.2 confirms this hypothesis as FL in activated sludge microcosms remained constant in FL 100 µg L^{-1} microcosm during the first 2 h of the experiment. In the case of FL 10 µg L^{-1}, a significant drop of FL concentration took place during the first 0.5 h (likely due to sorption) and then remained stable during the first 3 h of the experiment, showing a significant lag phase.

Several reports utilized pseudo-first-order kinetics for degradation of micropollutants in activated sludge reactors (Joss et al. 2006, Suarez et al. 2010, Collado et al. 2012). Indeed when applying pseudo-first order kinetics (OECD 303) in this work, $\ln(C_e/C_i)$ plotted as a function of time yielded a straight line (R^2>0.9). Pseudo-first order biodegradation rate k_1 [L $g^{-1}h^{-1}$] (normalised for concentration of suspended solids) was therefore calculated using the following formula (equation (9.1)):

$$ln\frac{C_e}{C_i} = -k_1 * t * SS \qquad (9.1)$$

where: t = aeration time (24h), C_e = concentration at time point t (µg L^{-1}), C_i = initial concentration (µg L^{-1}), SS = concentration of activated sludge solids (g L^{-1}).

2.4. Analysis

2.4.1. Solid phase extraction

Samples (50 mL of river water and 100 mL of activated sludge) were filtered using Whatman GF/F 0.7 µm glass fiber filter. Samples were concentrated using SPE. Oasis HLB cartridges (60 mg, 3 mL) were conditioned with 2 mL of MeOH and equilibrated with 2 mL of HQ water at a rate of 3 mL min^{-1}. Samples were passed through the HLB cartridge at a rate of 8 mL min^{-1} and then dried under the vacuum for 30 min to dry out residual water. Analytes were eluted with 4 mL of MeOH at a rate of <1mL min^{-1}. Extracts were then evaporated to dryness with TurboVap evaporator (Caliper, UK, 40°C, N_2, <5psi) and reconstituted in 0.5 mL of mobile phase. All samples were filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13 mm) and transferred to popylpropylene 0.3 mL capacity vials (Waters, UK).

SPE recoveries of FL and NFL in HQ water, river water and activated sludge were calculated as the ratio of the analyte peak area in the sample extract spiked with analytes before extraction (the peak area of analyte unspiked sample extract was subtracted) to the analyte peak area in the non-extracted standard solution.

Matrix effect (ME) was calculated for each chiral drug as a percentage decrease or increase in signal intensity in a sample matrix versus HQ water using the following equation (9.2):

$$ME = \frac{\Delta matrix}{\Delta HQ\ water} \qquad (9.2)$$

Where Δmatrix is the standard calibration graph slope in different matrix (river water or active sludge) and ΔHQ water is the standard calibration graph slope in HQ water.

2.4.2. Chiral-LC-MS/MS method development

2.4.2.1. Chiral liquid chromatography

Chromatographic analysis was performed using an Acquity UPLC system (Waters, Manchester, UK) consisting of Acquity UPLC binary solvent manager and Acquity UPLC sample manager. To achieve suitable separation of FL and NFL and their two stereoisomers, two chiral columns, namely Chiralpak CBH (10 cm × 2.0 mm, 5 µm particle size) and Astec Chirobiotic V (CBV; 25 cm × 2.1 mm, 5 µm particle size) were screened.

Several mobile phases were tested in order to obtain chiral separation of FL ad NFL using LC and to maintain satisfactory electrospray ionization (ESI) performance in the positive ionization mode. MeOH, EtOH and HQ water were used at different concentrations as the mobile phase solvents. Among the mobile phase additives, different concentrations of AAC (1, 4 and 10 mM), MeOH (85, 80 and 70%) and EtOH (70 and 80%) were tested to maximize the resolution of enantiomers (R_s) of FL and NFL.

The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. R_s was calculated using the following equation (9.3):

$$R_S = \frac{2\,(tr_{E2} - tr_{E1})}{W_{E2} + W_{E1}} \qquad (9.3)$$

where tr_{E1} and tr_{E2} are the retention times of the first and the second eluted enantiomers, respectively, and W_{E1} and W_{E2} are the widths of these responses at the base peak.

The best enantiomeric separation of the studied drugs was achieved with a mobile phase (pH 6.5) composed of 70% of EtOH, 30% HQ water, 4 mM of AAC and 0.005% of FA using a CBV column. The separation of enantiomers of chiral pharmaceuticals was undertaken under isocratic conditions, with an injection volume of 20 µL. The column was kept at 25°C and the temperature in the sample manager was

kept at 4°C. The flow rate was 0.06 mL min^{-1}, which gave an initial pressure of ~850 psi.

2.4.2.2. Chiral-LC-MS/MS conditions

Identification and quantification of FL and NFL was undertaken with an Acquity Xevo TQD (Waters, Manchester, UK), a triple quadrupole MS equipped with an electrospray ionization source. The analyses were performed in positive mode with a capillary voltage of 3 kV, a source temperature of 150°C and a desolvation temperature of 250°C. A cone gas flow of 50 L h^{-1} and desolvation gas flow of 450 L h^{-1} were used. Nitrogen, used as a nebulising and desolvation gas, was provided by a high purity nitrogen generator (Peak Scientific Instruments Ltd., UK). Argon (99.99%) was used as a collision gas. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, measuring the fragmentation of the protonated pseudo-molecular ions of each compound. A dwell time of 20 ms per ion pair was optimized to maintain high sensitivity of the analysis. MassLynx v4.1 (Waters, UK) software was used to collect and analyze the obtained data.

Quantifier and qualifier transitions were optimized for each compound based on the most intense signal. Specific parameters such as collision energy (CE) and cone voltage (CV) were optimized for FL, NFL and FL-d5 separately in a continuous flow mode through direct injection of standard solution at a concentration of 50 µg L^{-1} into the stream of the mobile phase. FL presents a precursor ion $[M+H]^+$ m/z of 310.3 and a product ion of m/z 44.2 (quantifier transition) with a CV of 34 and CE of 10 and m/z 148.2 (qualifier transition) with a CV of 25 and CE of 8. NFL presents a precursor ion $[M+H]^+$ m/z 298.4 and a corresponding product ions at m/z 134.1 (quantifier transition) with a CV of 17 and CE of 7 and m/z 30 (qualifier transition) with a CV of 17 and CE

of 7 . FL-d5 presents a precursor ion $[M-H]^+$ m/z 315.2 and a corresponding product ions at m/z 136.2 (quantifier transition) with a CV of 26 and CE of 71 and m/z 20.

2.4.3. Method validation parameters

Quantification of chiral compounds was carried out by means of MRM. The most abundant product ion of each target analyte was used for quantification and the second most abundant product ion was used for confirmation. A 10-point multi-component IS calibration curve was applied for quantification of FL and NFL enantiomers.

All instrumental and method validation parameters such as linearity and range, accuracy, precision, detection and quantification limits and calibration curve were determined for HQ water, river water and activated sludge spiked with known concentrations of chiral compounds.

Linearity and range of the analytical procedure were undertaken by serial dilution of stock solution (10 $\mu g\ mL^{-1}$).

Accuracy of the method was evaluated as a percentage deviation from the known added quantity of each enantiomer in the sample. Precision was expressed by the relative standard deviation (RSD) of 3 replicate measurements. Instrumental intra-day and inter-day precision were determined by analyzing 3 concentrations (5, 25 and 250 $\mu g\ L^{-1}$ of each enantiomer) of standards on the same day (3 replicates) or different days, respectively. Intra-day and inter-day of the analytical method were verified under the same operating conditions on the same day (3 replicates) or on 3 different days, respectively covering 3 concentrations (5, 25 and 250 of each enantiomer) of standards.

HQ water standard solutions were used for instrumental detection and instrumental quantification limits determination (IDL and IQL, respectively). The

quantification limit (QL) was estimated for the concentration of compound that gave a signal-to-noise ratio of 10:1. The detection limit (DL) corresponded to the concentration that gave a signal-to-noise of 3:1.

Method detection limits (MDL) and method quantification limits (MQL) for river water and active sludge were calculated using the following equations (9.4) and (9.5):

$$MDL = \frac{IDL_{S/N} \times 100}{Rec \times CF} \quad (9.4)$$

$$MQLcalc = \frac{IQL_{S/N} \times 100}{Rec \times CF} \quad (9.5)$$

where $IDL_{S/N}$ is the instrumental detection limit (µg L^{-1}), $IQL_{S/N}$ is the instrumental quantification limit (µg L^{-1}), Rec is the absolute recovery of the analyte (%) at 25 µg L^{-1}, and CF is the concentration factor, which in this method denotes 200 for active sludge and 100 for river water.

The enantiomeric fraction (EF) of studied chiral drugs was calculated using the following equation (9.6):

$$EF = \frac{E1}{E1+E2} \quad (9.6)$$

Where E1 and E2 are concentrations for the first (E1) and the last (E2) enantiomer of a chiral drug eluting from the CBH column. The injection of the single enantiomer standards ((*S*)-FL, (*R*)-FL, (*S*)-NFL and (*R*)-NFL) was performed to define the elution order of the enantiomers. In the case of FL and NFL, E1 and E2 enantiomers were identified as the (*S*)- and (*R*)- enantiomers, respectively.

2.5. Toxicity tests

2.5.1. *Daphnia magna* acute 48 h Immobilization Test.

The *D. magna* bioassay was carried out using the commercially available Daphtoxkit^{TM} (Crustacean Toxicity Screening Test for Freshwater; Microbiotests, Nazareth, Belgium) following the standard operational procedure in accordance to the ISO standard 6341:2012 and the OECD 202 guideline. Less than 24 h old daphnids were exposed to a series of concentrations of each enantiomer of FL and NFL. Six concentration levels (5 concentrations plus control, four replicate beakers for each concentrations, five individual for each beaker) were tested. The concentrations were from 0.5 to 50 mg L^{-1} for FL and NFL enantiomers based on preliminary range finding tests. Each experiment was repeated in triplicate. After 48 h incubation, daphnids were observed and the mobile daphnids in each container were reported. The median effective concentration values (EC50) were calculated using 48 h results.

2.5.2. Protozoa

These toxicity tests were carried out using the commercially available Protoxkit F, which was acquired from Microbiotests, Ghent University, Belgium. The tests were performed in accordance with the protocols provided by the manufacturer. Protoxkit F is a 24 h protozoan population growth assay. It is a chronic toxicity assay despite the short duration of the tests due to the short reproductive cycle of the protozoa, *T. thermophila*. This organism is particularly suited for this work due to their sensitivity to many environmental contaminants, their position in the ecosystem, potential for bioaccumulation and active presence in wastewater treatment processes.

The tests were carried out in disposable spectrophotometric cells of 1 cm path-length, to enable the measurement of the optical density (OD) at 440 nm. These measurements were taken at T0h and T24h, as well as two hour increments after this initial 24h incubation to monitor the change in turbidity of the sample. The reconstituted food substrate supplied with each test provides an initial high turbidity at T0h, which, in

the control cells, drastically decreases over the next 24 h due to the uninhibited growth of the ciliate population. This change in OD over the time period is used to quantify the degree of inhibition and subsequent calculation of the EC50. Each concentration was repeated in duplicate.

The initial protozoa inoculum was prepared by measuring an aliquot of the live suspension using photometry absorbance at 440 nm and diluted to achieve a theoretical OD value of 0.040. Each test cell is inoculated with 40 µL of this suspension, lending to an approximate population density of 100 protozoa per milliliter.

An initial study was carried out to find the approximate range of uninhibited growth and 100% inhibition for each enantiomer/compound across 7 orders of magnitude. The definitive toxicity test was carried out from the lowest concentration with a percentage population growth inhibition of 80-100% to the highest concentration with an inhibition between 0-20%. To ensure the test was valid the control must reach 60% OD decrease after 24 h. In some tests this may take 2 – 4h longer than 24h, this is batch dependent and indicates a slightly slower growth of the ciliates, however this is still considered valid. In this case all the tests carried out were with the same batch which took 28 h to fulfill the validation criteria. The EC50 values were calculated using 28 h results.

3. Results

3.1. Enantioseparation of FL and NFL enantiomers - method development and validation

The best enantiomeric separation of FL and NFL was achieved using a CBV column with mobile phase (pH 6.5) composed of 70% of EtOH, 30% of HQ water, 4

mM of AAC and 0.005% of FA. The enantiomeric separation was undertaken under isocratic conditions. The optimal flow rate was 0.06 mL min^{-1} with injection volume 20 µL. The column temperature was 25°C. Under these conditions baseline separation of enantiomeric pairs was achieved (**Figure 9.2**) and the R_s denoted 1.41 and 1.00 for FL and NFL respectively. The obtained retention times were 35.37 min for (*S*)-FL, 39.55 min for (*R*)-FL, 33.79 min for (*S*)-NFL and 36.12 min for (*R*)-NFL (see **Table S9.4** in the supplementary information for all conditions tested and results).

The method showed good linearity ($R^2 > 0.99$) for all four enantiomers within the studied range (0.25-100 µg L^{-1}). $IDL_{S/N}$ and $IQL_{S/N}$ were 0.12 and 0.5 µg L^{-1} for (*S*)-FL, (*R*)-FL, (*S*)-NFL and (*R*)-NFL respectively. Furthermore, MDLs and MQLs for river water matrices ranged from 1.2 to 1.3 ng L^{-1} and from 4.6 to 5.1 ng L^{-1}, respectively. In the case of activated sludge matrices, MDLs ranged from 0.4 to 0.8 ng L^{-1} and MQLs ranged from 1.7 to 3.1 ng L^{-1} (**Table S9.5** in the supplementary information). The accuracy was within ± 20%. Both intra- and inter-day repeatability, as indicated by RSD calculated from the analysis of 3 replicates, was on average less than 20% (**Table S9.6** in the supplementary information).

Absolute recoveries were obtained for studied chiral drugs in HQ water, river water and activated sludge. Very good recoveries accounting for > 67% were observed in the case of all four enantiomers in all studied matrices. Furthermore, the extraction process of chiral drugs on HBL cartridges was found not to be enantioselective. ME for each enantiomer was minimal and was observed to be less than 15.6%.

3.2. Transformation of FL and NFL in river and activated sludge simulating microcosms

3.2.1. River water microcosms

The river simulating microcosms revealed that degradation of FL takes place via both microbial and photochemical processes (**Figure 9.3** and **Table S9.7** in the supplementary information). Photolysis is considered to be the most important phenomenon contributing to the degradation of FL, as 74.5% ((*S*)-FL) and 79.2% ((*R*)-FL) of FL were removed in light abiotic conditions (LAR). This process, as expected, was found not to be stereoselective. Microbial processes resulted in mild enantioselectivity towards (*R*)-enantiomer and led to the removal of 60.4% ((*S*)-FL) and 67.9% ((*R*)-FL) at dark biotic conditions (DBR). As expected, the light biotic conditions reactor (LBR) utilizing both photochemical and microbial processes led to the highest removal of FL: 98.4% of (*S*)-FL and 96.7% of (*R*)-FL. Dark abiotic conditions (DAR) did not lead to any degradation of FL which indicates no significant contribution of sorption of FL to e.g. suspended particulate matter. Traces of NFL were observed in both abiotic and biotic conditions (**Figure S9**.5 in the supplementary information). This indicates that degradation of FL leading to NFL formation takes place during both photochemical and microbial processes.

3.2.2. Activated sludge microcosm

Transformation of FL in activated sludge simulating microcosms was studied at two concentration levels: 10 and 100 µg L^{-1} of racemic FL (**Figure 9.4** and **Table S9.8** in the supplementary information). In both cases a significant decrease in the concentration of (*S*)-FL and (*R*)-FL was observed. In the microcosm spiked with 10 µg L^{-1} rapid removal of FL occurred during the first 30 minutes (50% degradation). This process was not stereoselective (EF, 0.5) and did not lead to expected formation of NFL. It is therefore postulated that this high rapid removal of FL from the aqueous phase during the first 30 min of the experiment is due to its sorption to suspended particulate matter. Further removal of FL in 10 µg L^{-1} reactor was much slower and led

to its stereoselective transformation favoring (*S*)-FL (EF, <0.3) and leading to the formation of NFL enriched with (*S*)-enantiomer (EF, >0.7). As the activated sludge simulating microcosms were undertaken in the dark, it is postulated that observed stereoselective transformation of FL and stereoselective formation of NFL is due to the prevalence of stereoselective microbial metabolic processes in studied bioreactors. Molar percentage yield of NFL formation denoted: 10.7% and 6.2% for (*S*)-FL and (*R*)-FL respectively.

Similar observations were recorded in the microcosm spiked with 100 μg L^{-1} of FL. However, the effect of sorption was not observed. This is probably due to much higher initial FL load in 100 μg L^{-1} bioreactor not allowing for the change to be recorded. Stereoselective microbial processes resulted in 60% transformation of FL with twice as high preference towards (*S*)-FL (EF, <0.3, 80% removal of (*S*)-FL and only 38% removal of (*R*)-FL) and formation of NFL enriched with (*S*)-enantiomer (EF, >0.7). Molar percentage yield of NFL formation denoted: 11.7% and 7.4% for (*S*)-FL and (*R*)-FL. This is in agreement with results obtained for 10 μg L^{-1} bioreactor. Interestingly in both bioreactors, long lag phases (3h and 2h in the case of 10 μg L^{-1} and 100 μg L^{-1} FL bioreactor respectively) were observed.

Kinetic studies (**Table 9.1**) confirmed low biodegradation of FL and the more recalcitrant nature of (*R*)-FL. k_{biol} and $t_{1/2biol}$ of (*S*)-FL transformation were 0.04 $Lg_{SS}^{-1}h^{-1}$ and 19h respectively in both 10 and 100 μg L^{-1} bioreactors. k_{biol} and $t_{1/2biol}$ of (*R*)-FL transformation were much lower and denoted 0.01 $Lg_{SS}^{-1}h^{-1}$ and 68h respectively in 100 μg L^{-1} bioreactors. Due to the lack of degradation of (*R*)-FL in 10 μg L^{-1} bioreactor, no kinetic studies could be undertaken.

The results above indicate that longer sludge retention times are needed during wastewater treatment in order to facilitate degradation of FL. However, it should be

noted that these processes are likely to be stereoselective and could potentially lead to enrichment of FL with the more potent enantiomer, as well as formation of biologically active metabolites; this is despite the nominal decrease in concentration levels of FL.

As mentioned above, biodegradation of FL during activated treatment process favors the (*S*)-enantiomer, which leads to the enrichment of FL with the (*R*)-enantiomer. This is in contrast with metabolic processes in humans, which favor the (*R*)-enantiomer and lead to enrichment of FL in urine with the (*S*)-enantiomer (Caccia, 1998). Indeed in our full scale untreated wastewater study, FL was enriched with the (*S*)-enantiomer (EF, 0.68) (Petrie et al. 2016). Several other studies reported FL to be enriched with the (*S*)-enantiomer in wastewater (EF, >0.60) and in contaminated rivers (Barclay et al. 2012a, López-Serna et al. 2013, Evans et al. 2015a, Ma et al. 2016). Barclay et al 2012 published no significant stereoselectivity was observed during some wastewater treatment processes (Barclay et al. 2012a). Nevertheless Ribeiro et al. 2014 reported that (*R*) FL was detected in effluents of WWTP indicating a faster degradation of (*S*) FL during the biological degradation occurring at those WWTP. These findings were in accordance with Mc Leod et al. (2007) who verified an enrichment of in (*R*) FL (MacLeod et al. 2007, Ribeiro et al. 2014). The outcomes of human metabolism studies as well as full scale and microcosm wastewater treatment measurements indicate that enantiomeric signature of FL can change subject to composition and structure of microbial communities present in wastewater. This confirms, yet again, complexity of environmental processes and reinforces the need for further comprehensive studies focusing on transformation of chiral pollutants in the environment.

3.3. Ecotoxicity of FL and NFL

FL is known to be toxic to several aquatic species at low environmentally relevant concentrations (**Table 9.2**) (Brooks et al. 2003, Foran et al. 2004, De Andrés et

al. 2009, Gonzalez-Rey and Bebianno 2013). Both FL and NFL are included in the list of 10 pharmaceuticals that are potentially dangerous for the environment (Ribeiro et al. 2014). Despite their different potency of FL and NFL enantiomers and their different environmental fates, very limited work has been undertaken to verify if toxicity of FL and NFL is enantiomer-dependent.

Stanley at al. (2007) (Stanley et al. 2007) assessed potential enantiospecific differences in sublethal standardized and behavioral responses of *P. promelas* (7 days tests) and *D. magna* (21 days tests) to FL. The following endpoints were assessed: immobilization, reproduction, and grazing rate in *D. magna* and survival, growth, and feeding rate in *P. promelas*. (*S*)-FL was found to be more toxic to *P. promelas*, potentially because its primary active metabolite, (*S*)-NFL, is more potent than the same metabolite of (*R*)-FL in mammals. Interestingly, no enantioselectivity was observed in acute 42 h tests in *P. promelas.* In contrast to *P. promelas* study (over 7 days), no enantioselectivity was observed in *D. magna*. This differential enantiospecific response between model organisms may have resulted from closer target homology between mammals and fish than between mammals and crustaceans (Stanley, Ramirez et al. 2007). De Andres et al. (2009) reported EC50 of 30.5 mg L^{-1} for (*R*)-FL and 3.2 mg L^{-1} for (*R*)-FL to *T. thermophila* (De Andrés et al. 2009). These values indicate certain enantioselectivity of the protozoan to these enantiomers with the sensitivity to (S) -FL.

In this study, $EC50_{48h}$ for FL enantiomers towards *D. magna* was 3.6 mg L^{-1} and 4.1 mg L^{-1} for (*S*)-FL and (*R*)-FL respectively. These values are in the same order of magnitude as EC50 reported by Minguez et al. (2014) (Minguez et al. 2014) and Christensen et al. (2007) (Christensen et al. 2007) and are in good agreement with other reports: De Andrés et al. (2009) (De Andrés et al. 2009). $EC50_{48h}$ for NFL towards *D. magna* denoted 2.8 mg L^{-1} and 2.9 mg L^{-1} for (*S*)-NFL and (*R*)-NFL (raw data are

shown in **Table S9.9** in the supplementary information). The results indicate an obvious difference between the toxicity of FL and NFL, NFL being more toxic than FL, but no significant enantioselectivity was observed for studied enantiomeric pairs.

Furthermore, $EC50_{24h}$ for FL enantiomers towards *T. thermophila* denoted 35.3 mg L^{-1}, 1.3 mg L^{-1}, and 0.9 mg L^{-1} for (*S*)-FL, (*R*)-FL and a mixture of the FL enantiomers (EF, 0.3) respectively. These results contradict those published by De Andres et al. (2009) as within that study it was observed that the (*S*)-enantiomer was more toxic with an $EC50_{24h}$ of 3.2 mg L^{-1} compared to the (*R*)-enantiomer with an $EC50_{24h}$ 30.5 mg L^{-1}. For clarity the stock solutions and the test cells were analysed to confirm the concentration before and after the test and to investigate if any changes were observed during the test. The results showed that the changes in the concentrations of the toxicants were minimal during the test, however they support the use of the correct enantiomer in this test. This was further confirmed by the use of enantiomerically pure analytical standards (purchased from Sigma) to confirm the RT of each enantiomer (**Figure S9.6** in the supplementary information).

These results also suggest a synergistic effect between the two FL enantiomers in the non-racemic mixture (EF, 0.3), although this will need to be confirmed and explored with further studies. There is to date, no other study which has compared the $EC50_{24h}$ of the enantiomers and a mixture of the two. The $EC50_{24h}$ for NFL denoted 3.8 mg L^{-1}, 5.9 mg L^{-1}, 0.5 mg L^{-1} for (*S*)-NFL, (*R*)-NFL and a mixture of the NFL enantiomers (EF, 0.3). Unlike FL the (*S*)-enantiomer is more toxic for NFL. (*R*)-NFL is four times less potent than (*R*)-FL. The $EC50_{24h}$ of the non-racemic mixture (EF, 0.3) NFL enantiomers also suggests a synergistic effect. A final test was carried out containing a mixture of the FL and NFL enantiomers (FL; EF, 0.3, NFL; EF, 0.3). The FL EF used in the test (FL; EF, 0.3) was based on an enrichment of the (*R*)-enantiomer

as seen in activated treatment processes. This is important consideration as this organism is part of the microbial community that takes part in this processes. **Figure 9.5** and **Tables S9.10-S9.23** in the supplementary information show the $EC50_{24h}$ data for *T. thermophila.*

The above results indicate that traditional toxicological studies that do not recognize the importance of stereochemistry might not reveal the true toxicological impact resulting from stereochemistry of chiral drugs. Our research indicates that (*S*)-FL is preferentially degraded in activated sludge microcosms. This is expected, as (*S*)-FL is the least toxic to protozoa (organisms that are known to be key contributors to activated sludge treatment process) out of all four FL/NFL enantiomers studied. Unfortunately, this also indicates that FL, due to preferential metabolic degradation of (*S*)-FL, gets enriched with more toxic (*R*)-FL. This accumulation of (*R*)-FL might have detrimental effects on the performance of activated sludge treatment processes.

One can assume that, if the stereochemistry of FL is not considered, decreased concentration of FL as a result of activated sludge treatment leads to decreased biological impact. Such an approach (as currently applied in ERA) can lead to false conclusions impacting environmental health. Our study proves that despite the overall decrease in FL concentration, accumulation of toxic (*R*)-FL and formation of toxic (*S*)-NFL in activated sludge will likely lead to higher toxicological effects, as observed in the case of protozoa.

4. Conclusions

This study is, to the authors' knowledge, the first to report transformation of FL in river and activated sludge simulating microcosms including effects of microbial degradation and photolysis. The research reported in this manuscript tested and

validated the hypothesis that degradation of FL, and formation of its main metabolite NFL, are stereoselective and biological in nature.

The river simulating microcosms revealed that degradation of FL takes place via both microbial and photochemical processes. Non-stereoselective photolysis was observed to be the most important phenomenon contributing to the degradation of FL. Microbial processes resulted in only mild enantioselectivity towards the (*R*)-enantiomer. However, a pronounced stereoselectivity was observed during activated sludge simulating microcosms. Microbial metabolic processes favoring (*S*)-FL led to enrichment of FL with the (*R*)-enantiomer and formation of NFL enriched with the (*S*)-enantiomer. This is in contrast to metabolic processes in humans, which favor the (*R*)-enantiomer and lead to enrichment of FL in urine with the (*S*)-enantiomer.

The outcomes of human metabolism studies, as well as full scale and microcosm wastewater treatment measurements, indicate that the enantiomeric signature of FL can change subject to composition and structure of microbial communities present in wastewater. This confirms, yet again, the complexity of environmental processes and reinforces the need for further comprehensive studies focusing on transformation of chiral pollutants in the environment.

Toxicity tests showed that while there is no significant enantioselectivity in the toxic response from *D. magna* to both FL and NFL, a strong enantiomer-dependent toxicity is observed in the case of *T. thermophila* ((*R*)-FL 30x higher than (*S*)-FL). Interestingly, in the case of NFL, the (*S*)-enantiomer exhibited higher toxicity than its corresponding (*S*)-FL enantiomer. Furthermore, when tested as mixtures, both FL and NFL showed higher toxicity than any of the enantiomerically pure standards.

This study revealed that there are several, unaccounted for, underlying issues in both exposure and hazard assessment within ERA of chiral pharmaceuticals. The

European Medicines Agency guideline on the ERA of Medicinal Products for Human Use (EMEA) and the EU Directive for ERA for Veterinary Medicinal Products (EEC) recommend the estimation of exposure and the prediction of risk calculation for the whole parent compounds only i.e. as a racemate or a mixture of stereoisomers if distributed as such. Therefore, current ERA leads to under or overestimation of toxicity of chiral pharmaceuticals and to incorrect environmental risk assessment as chiral pharmaceuticals are present in the environment in their non-racemic forms and they show enantiomer-specific biological effects. We therefore recommend the adoption of a new strategy within ERA acknowledging stereochemistry of studied targets.

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Tables

Table 9.1. Degradation pseudo-first order rate constants (k_1 and k_{biol}) in single-compound activated sludge simulating microcosm

		R^2	SS [g L^{-1}]	k_1 [h^{-1}]	$t_{1/2}$ [h]	k_{biol} [$Lg_{SS}^{-1}h^{-1}$]	$t_{1/2biol}$ [h]
10µg L^{-1}							
(S)-FL	y = -0.0715x - 0.0584	0.8696	2.0	0.07	9.69	0.04	18.9
(R)-FL	No degradation						
100µg L^{-1}							
(S)-FL	y = -0.0721x - 0.0667	0.8216	2.0	0.07	9.61	0.04	19.4
(R)-FL	y = -0.0206x - 0.0274	0.8202	2.0	0.02	33.6	0.01	68.0

n/a - not calculated due to no degradation of *(R)*-(-)-enantiomer

Table 9.2. Ecotoxicity of FL and its metabolite NFL (n/a – not analysed).

Organism	Test	Toxicity endpoints	Effect [mg L^{-1}]						References
			FL			NFL			
			(S)-	*(R)-*	*Rac*	*(S)-*	*(R)-*	*Rac*	
P. subcapitata	$EC50_{72h}$	-growth	n/a	n/a	0.20	n/a	n/a	n/a	(Minguez, Poi et al. 2014)
	$EC50_{24h}$	-IPAM	n/a	n/a	0.20	n/a	n/a	0.53	(Neuwoehner, Fenner et al. 2009)
		-growth	n/a	n/a	0.09	n/a	n/a	0.24	
	$EC50_{48h}$	-growth	n/a	n/a	0.03	n/a	n/a	n/a	(Christensen, Faaborg-Andersen et al. 2007)
	$EC50_{120h}$	-growth	n/a	n/a	0.02	n/a	n/a	n/a	(Brooks, Turner et al. 2003)
	$IC50_{96h}$	-growth	n/a	n/a	0.04	n/a	n/a	n/a	(Johnson, Sanderson et al. 2007)
S. acutus	$IC50_{96h}$	-growth	n/a	n/a	0.09	n/a	n/a	n/a	(Johnson, Sanderson et al. 2007)
S. quadricauda	$IC50_{96h}$	-growth	n/a	n/a	0.21	n/a	n/a	n/a	(Johnson, Sanderson et al. 2007)
Ch. vulgaris	$IC50_{96h}$	-growth	n/a	n/a	4.34	n/a	n/a	n/a	(Johnson, Sanderson et al. 2007)
H. attenuata	$EC50_{96h}$	-survival	n/a	n/a	7.94	n/a	n/a	n/a	(Minguez, Poi et al. 2014)
P. promelas	$LC50_{48h}$	-survival	n/a	n/a	0.71	n/a	n/a	n/a	(Brooks, Turner et al. 2003)
	$LOEC_{7d}$	-survival	0.10	0.17	0.17	n/a	n/a	n/a	(Stanley, Ramirez et al. 2007)
		-growth	0.05	0.17	0.05	n/a	n/a	n/a	
		-feeding	0.05	0.17	0.11	n/a	n/a	n/a	
	$LC50_{48h}$	-survival	0.22	0.21	0.20	n/a	n/a	n/a	(Stanley, Ramirez et al. 2007)
C. dubia	$LC50_{48h}$	-mortality	n/a	n/a	0.51	n/a	n/a	n/a	(Henry, Kwon et al. 2004)
	$LC50_{48h}$	-immobilization	n/a	n/a	0.23	n/a	n/a	n/a	(Brooks, Turner et al. 2003)
P. patulus	$LC50_{48h}$	-mortality	n/a	n/a	0.06	n/a	n/a	n/a	(Martinez Gomez, Baca et al. 2015)
D. magna	$EC50_{48h}$	-immobilization	n/a	n/a	6.4	n/a	n/a	n/a	(Christensen, Faaborg-Andersen et al. 2007)
	$EC50_{48h}$	-immobilization	n/a	n/a	5.91	n/a	n/a	n/a	(Minguez, Poi et al. 2014)
	$LC50_{48h}$	-immobilization	n/a	n/a	0.82	n/a	n/a	n/a	(Brooks, Turner et al. 2003)
	$LOEC_{21d}$	-immobilization	0.44	0.43	0.43	n/a	n/a	n/a	(Stanley, Ramirez et al. 2007)
		-reproduction	0.44	0.43	0.43	n/a	n/a	n/a	
		-grazing	0.20	none	none	n/a	n/a	n/a	
	$LC50_{48h}$	-immobilization	6.9	8.1	n/a	n/a	n/a	n/a	
	$EC50_{48h}$	-immobilization	3.6	4.1	n/a	2.8	2.9	n/a	(this study)
T. thermophila	$EC50_{24h}$	-growth	3.2	30.5	n/a	n/a	n/a	n/a	(DeAndrés et al. 2009)
	$EC50_{24h}$	-growth	35.2	1.3	0.5 (EF=0.3)	4.5	5.8	0.9 (EF=0.3)	(this study)

Figures

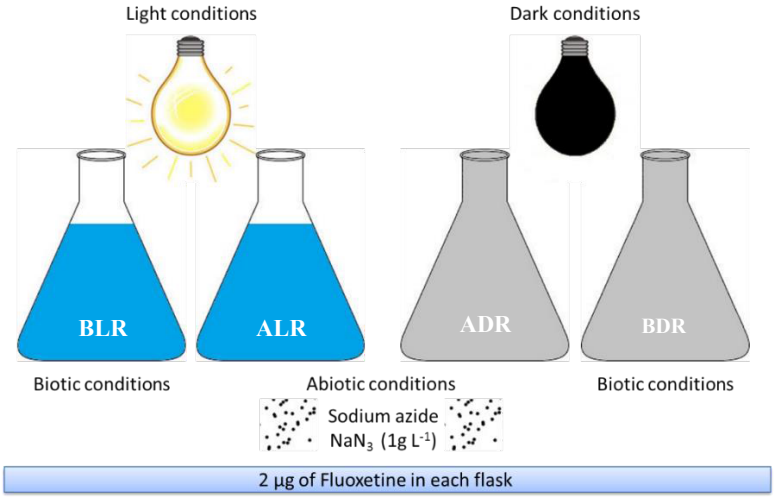

Figure 9.1 – Scheme of river and activated sludge simulating microcosms

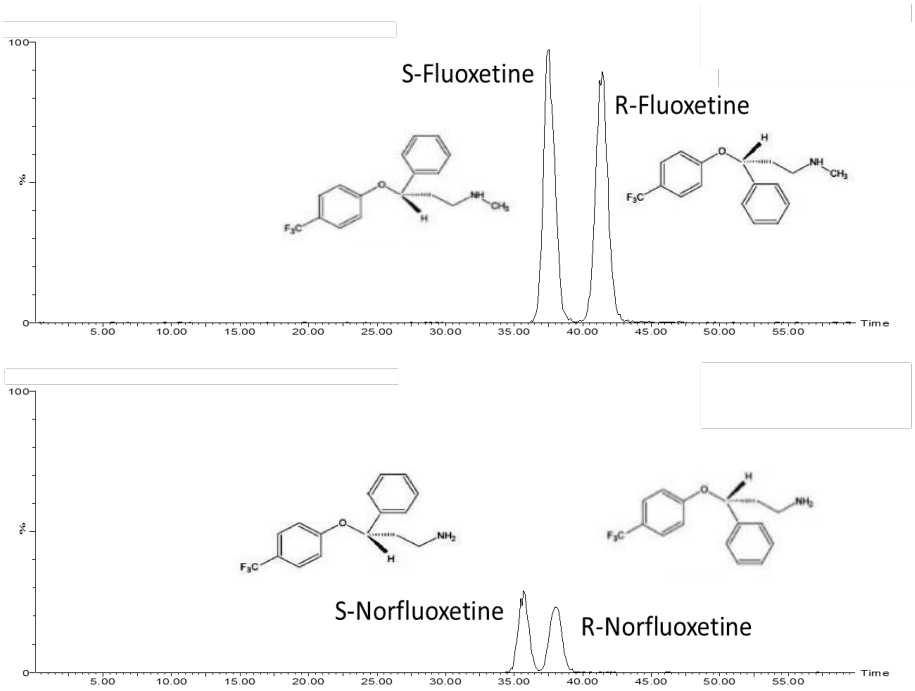

Figure 9.2 - Chromatographic separation of enantiomers of FL and NFL.

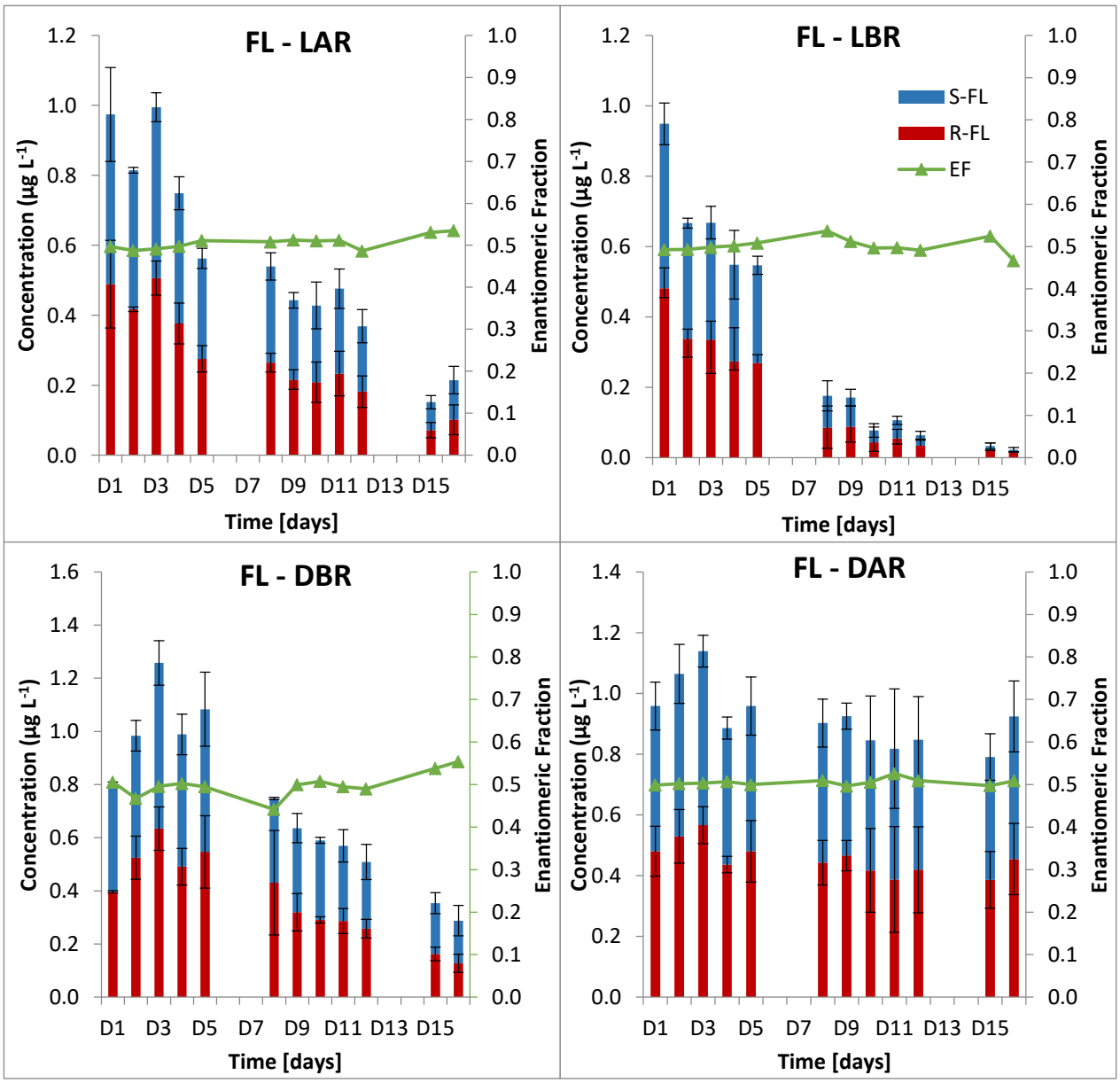

Figure 9.3 – Transformation of FL and formation of NFL in river water simulating microcosms under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols).

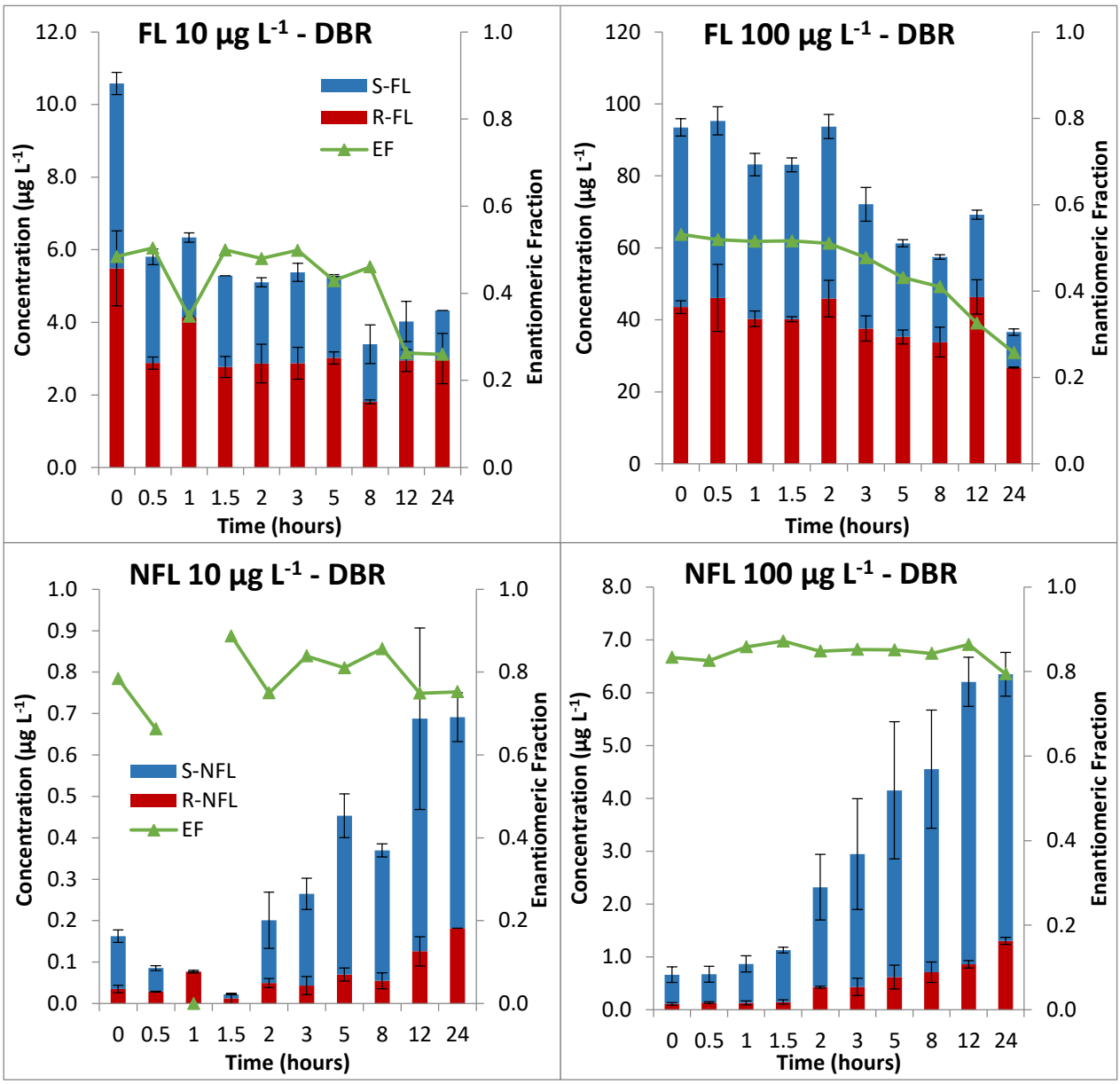

Figure 9.4 - Transformation of FL and formation of NFL in activated sludge simulating microcosms under dark biotic (DBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols).

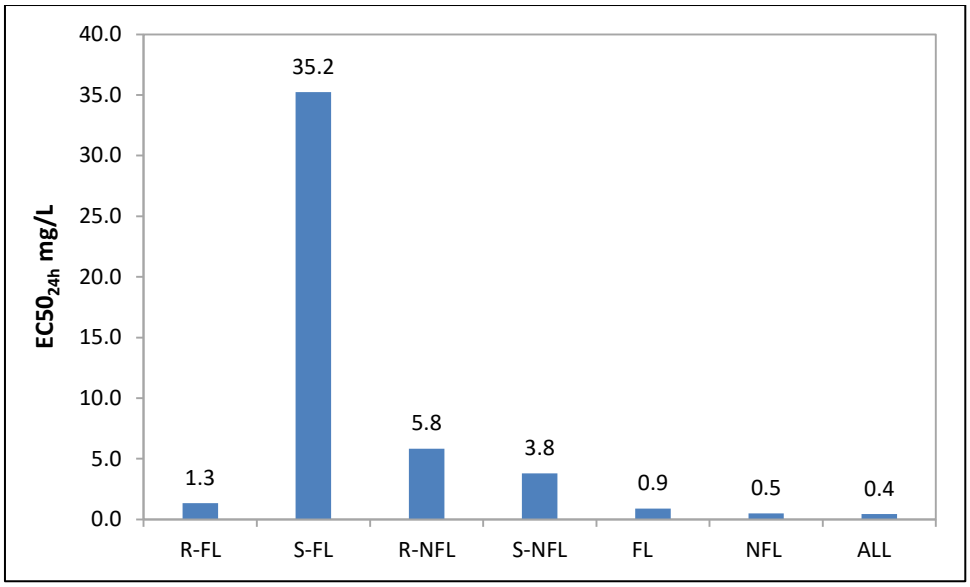

Figure 9.5 – $EC50_{28h}$ for the 7 test listed above: (*R*)-FL (EF = 0.0), (*S*)-FL (EF=1.0), FL (EF = 0.3), NFL (EF = 0.3), ALL ((*R*)-FL = 13%, (*S*)-FL = 7%, (*R*)-NFL = 54%, (*S*)-NFL = 26%; FL; EF=0.3, NFL; EF = 0.3). See Tabs 10-23 for CV% of individual tests.

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Supplementary Information

Enantioselective biodegradation of fluoxetine in water and its ecotoxicological relevance

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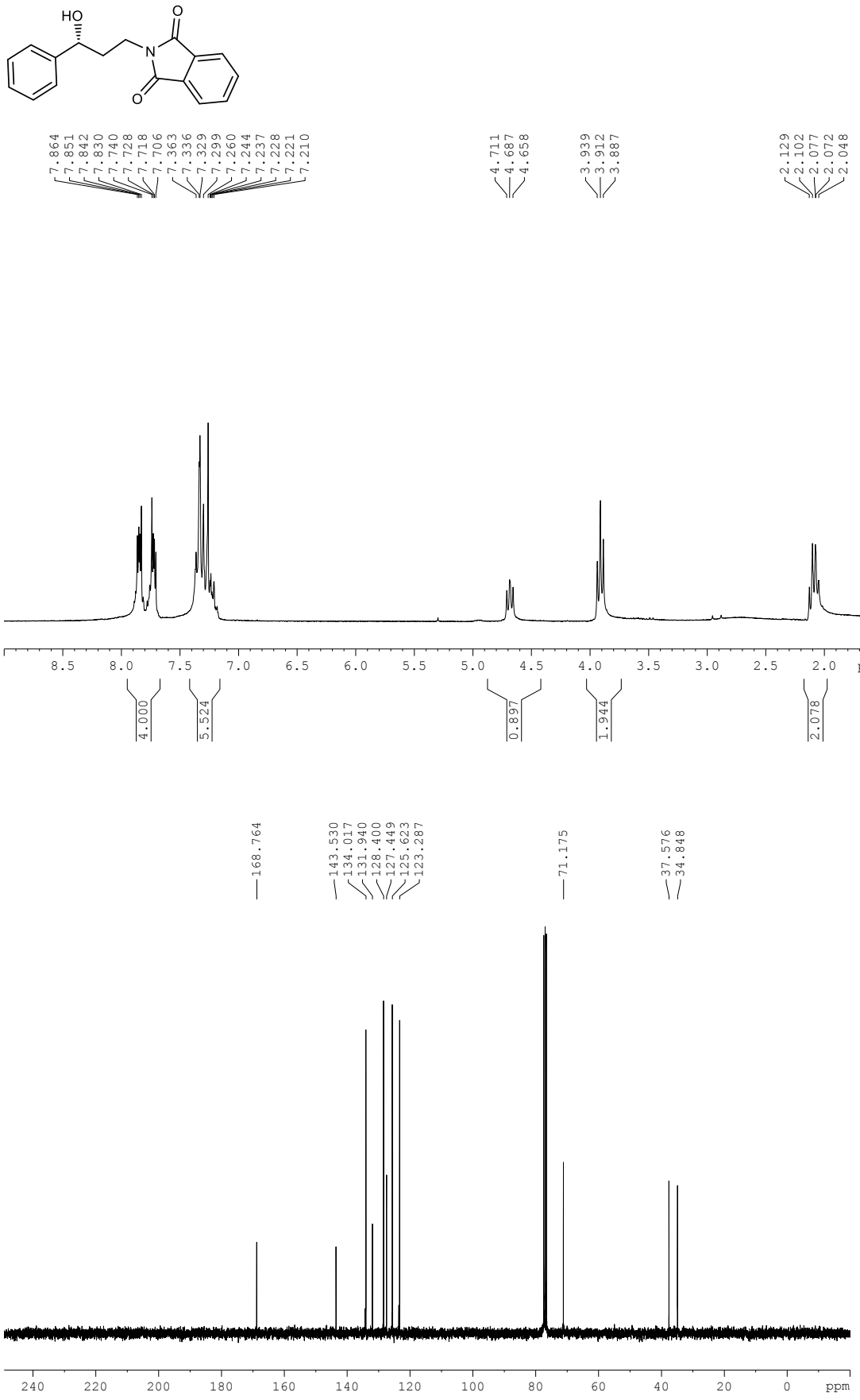


Figure S9.1. ^{1}H (upper) and ^{13}C NMR (down) average spectra of (*R*)-3-phthalimido-1-phenylpropanol.

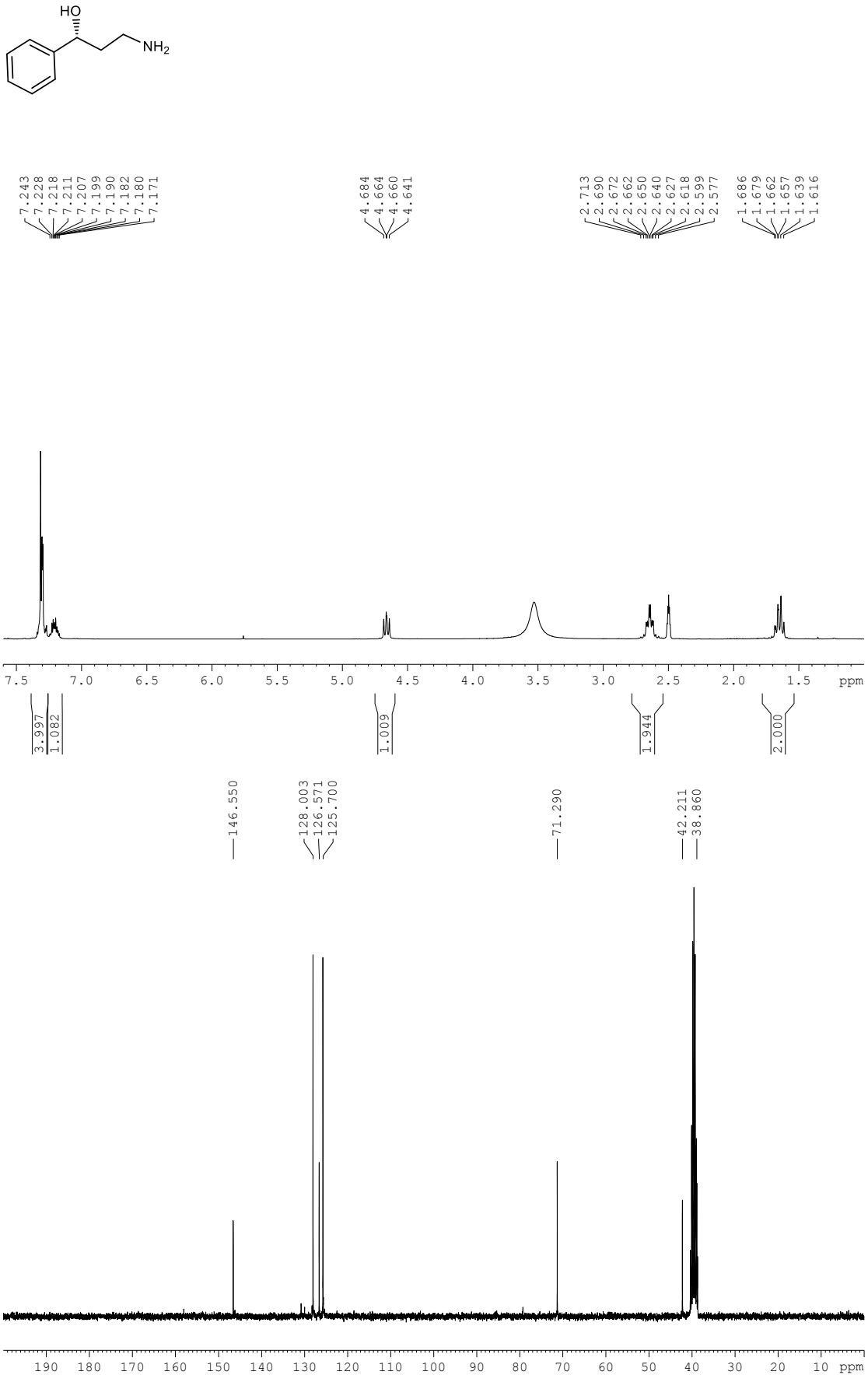


Figure S9.2. ^{1}H (upper) and ^{13}C NMR (down) average spectra of (*R*)-3-amino-1-phenyl-1-propanol.

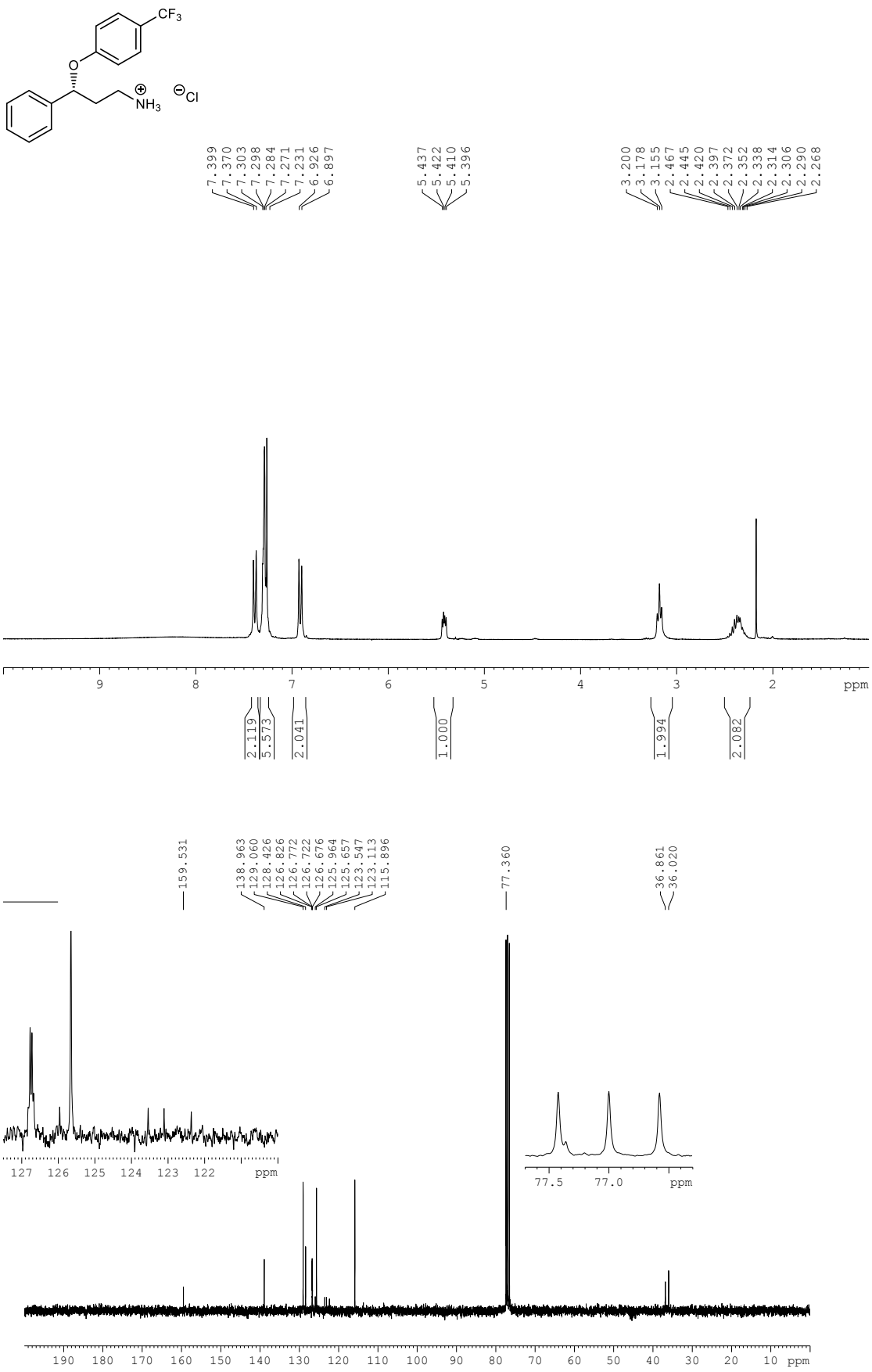


Figure S9.3. ^{1}H (upper) and ^{13}C NMR (down) average spectra of (*R*)-3-Phenyl-3-[4-(trifluoromethyl)phenoxy]-1-propanamine•HCl (*R*-NFL).

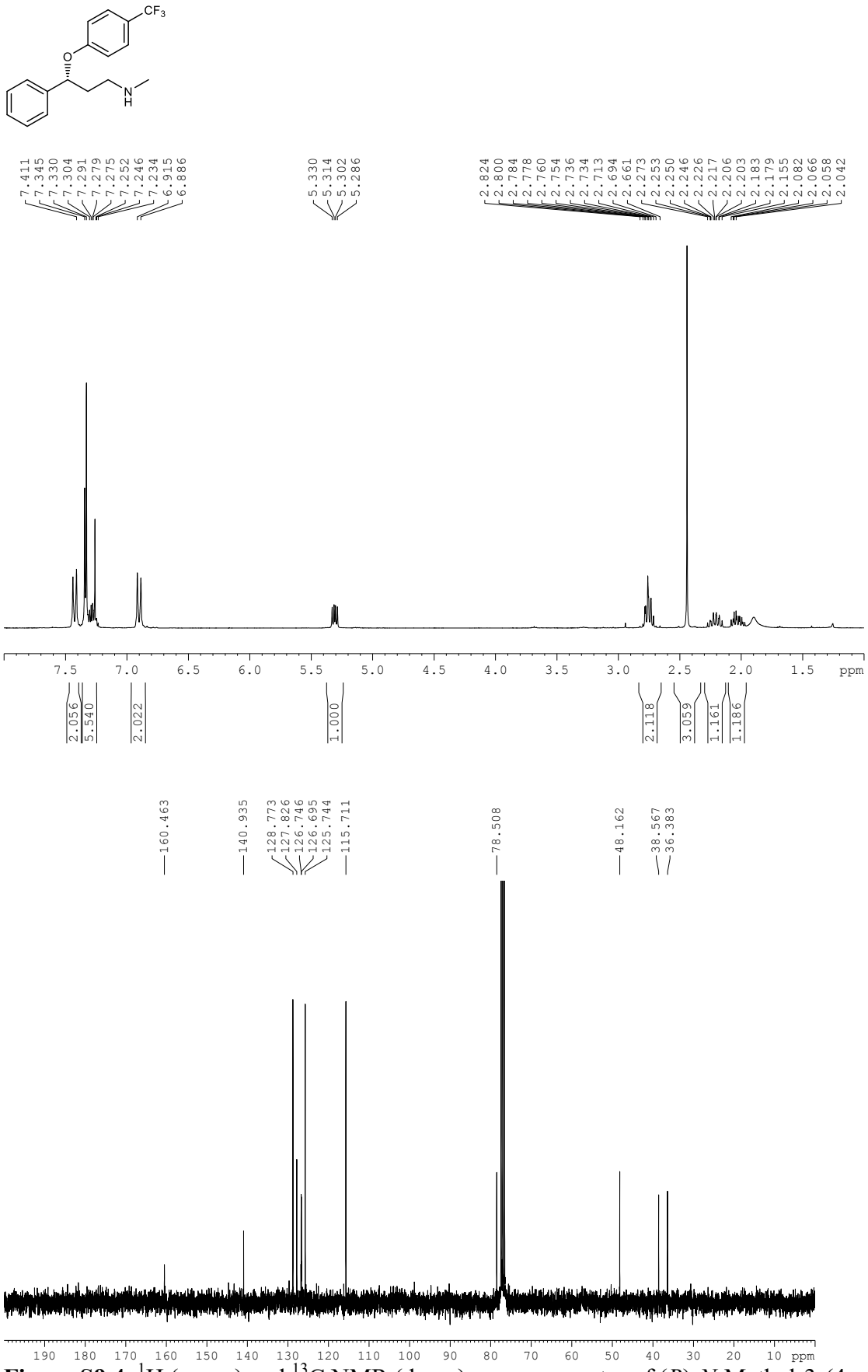


Figure S9.4. ^{1}H (upper) and ^{13}C NMR (down) average spectra of (*R*)-*N*-Methyl-3-(4-trifluoromethylphenoxy)-3-phenylpropylamine (*R*-FL).

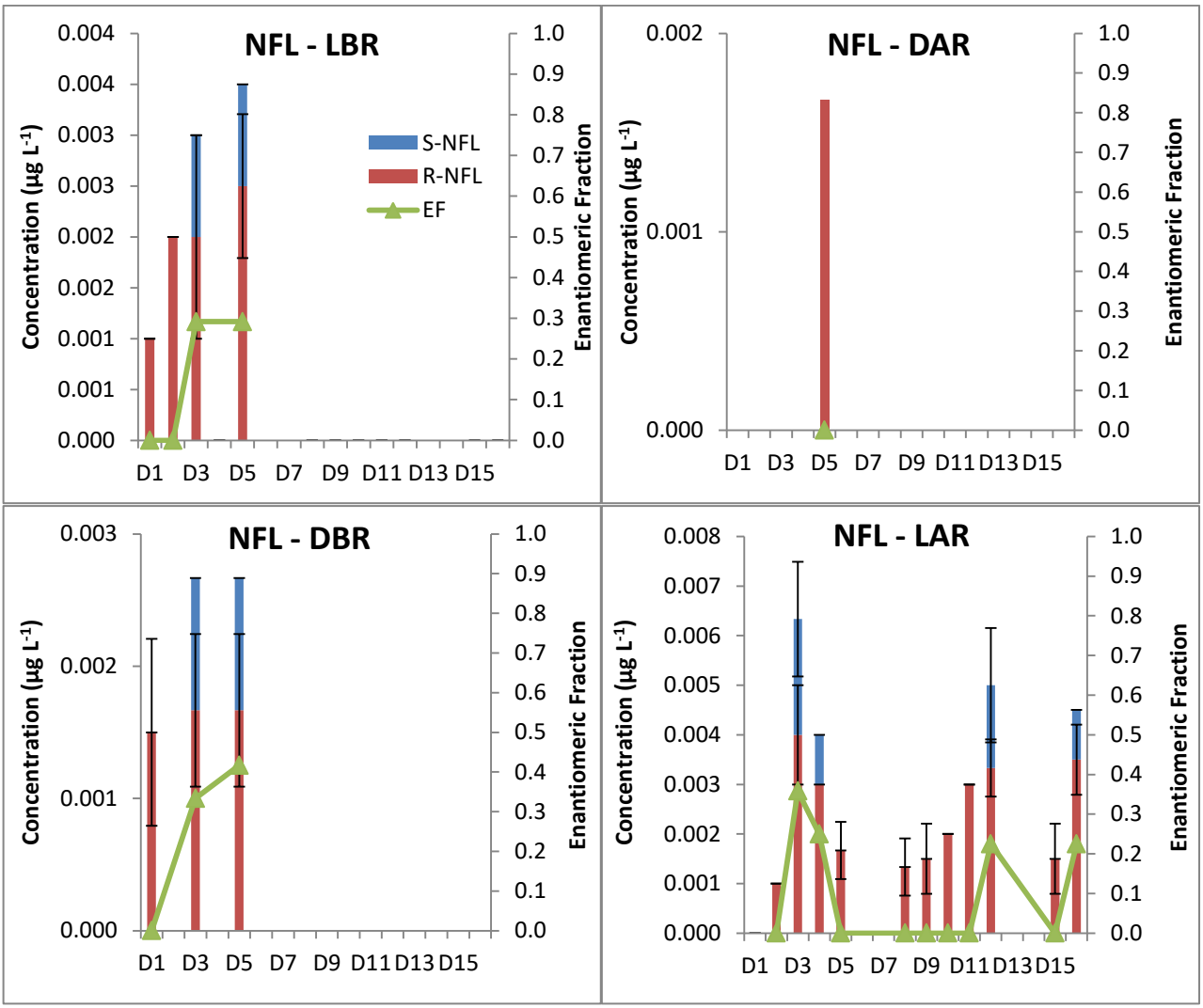


Figure S9.5. Formation of NFL during FL degradation in river simulating microcosms under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols).

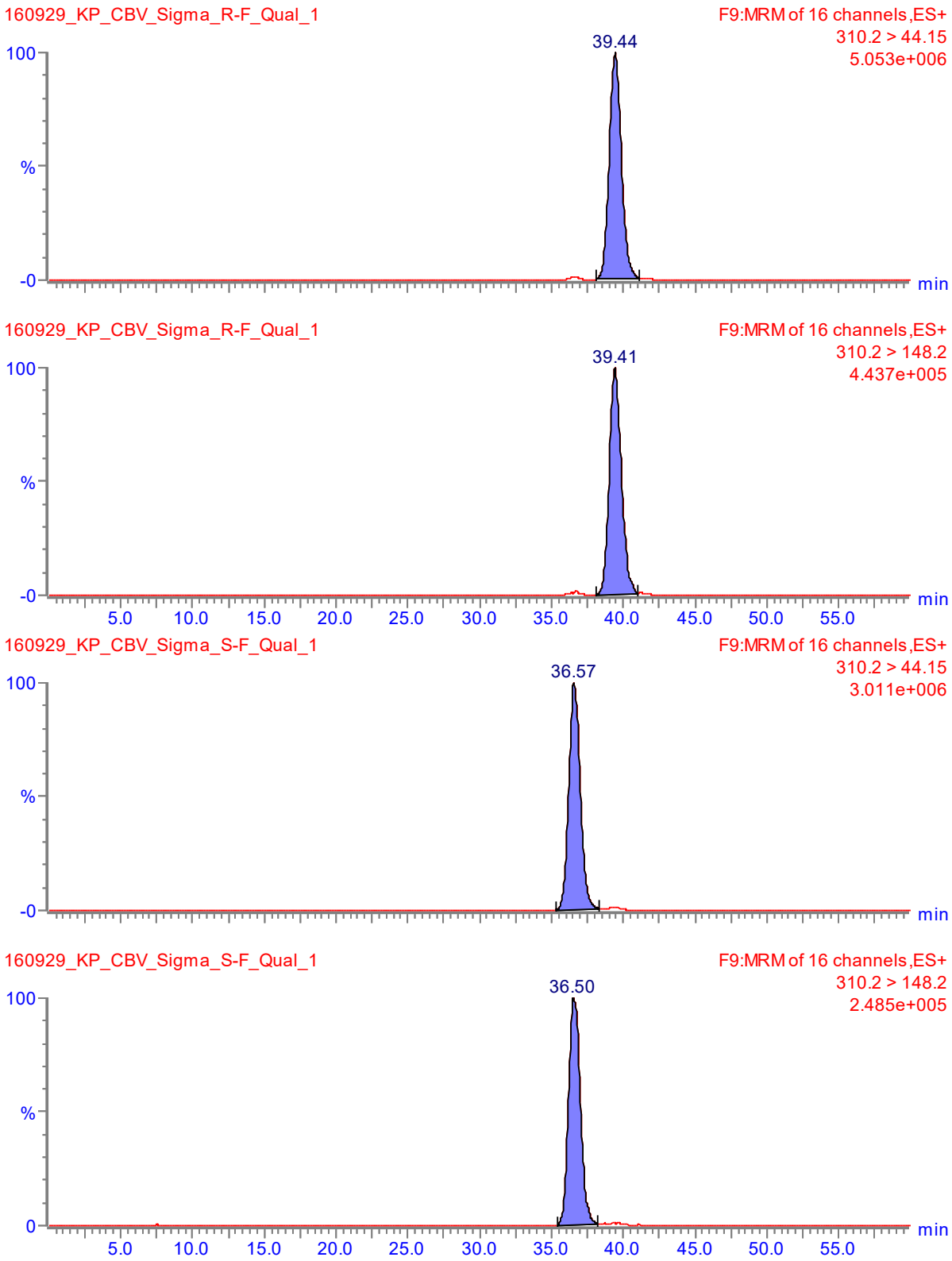


Figure S9.6. Qualitative confirmation of the retention time for Sigma enantiomerically pure FL standards. In order from top to bottom *R*-FL MRM1 RT = 39.44; *R*-FL MRM2 RT = 39.41; *S*-FL MRM1 RT = 36.57; *S*-FL MRM2 =36.50.

Table S9.1. Structures, molecular formula, molecular weight and pK_a of selected compounds.

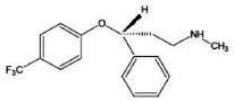
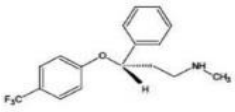
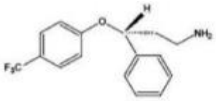
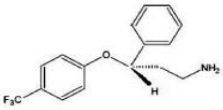
Compound	Chemical structure	Molecular formula	Mw (g mol^{-1})	pK_a
R-(-)-Fluoxetine (*R*-FL)		$C_{17}H_{18}F_3NO$	309.33	10.05
S-(+)-Fluoxetine (*S*-FL)				
R-(-)-Norfluoxetine (*R*-NFL)		$C_{16}H_{16}F_3NO$	295.30	9.05
S-(+)-Norfluoxetine (*S*-NFL)				

Table S9.2. Physicochemical parameters (dissolved oxygen (DO), pH, temperature, total suspended solids (TSS), NO_2^-, NH_4, and chemical oxygen demand (COD)) during the river water simulating microcosms under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions.

	D1	D2	D3	D4	D5	D8	D9	D10	D11	D12	D15	D16
pH												
DBR	8.16	8.07	7.41	7.32	7.41	7.94	8.14	8.06	7.85	7.91	8.40	8.47
DAR	8.31	8.58	8.74	8.88	8.49	8.20	8.15	7.91	8.00	7.95	8.38	8.38
LBR	8.40	8.79	7.98	7.92	7.70	8.20	8.08	8.33	8.07	8.31	8.41	8.46
LAR	8.38	8.84	8.94	8.97	8.38	8.36	8.27	8.46	8.14	8.44	8.40	8.42
DO (mg L^{-1})												
DBR	8.00	5.56	5.07	5.15	4.69	4.98	5.01	4.53	4.66	4.36	4.56	5.28
DAR	6.90	5.03	5.02	4.37	4.58	4.81	4.83	4.67	4.53	4.51	4.40	5.99
LBR	6.47	4.95	4.53	4.27	4.12	4.74	3.77	3.52	3.50	3.35	3.69	3.73
LAR	7.35	4.94	4.51	4.42	4.75	4.54	4.30	4.08	4.01	3.82	4.30	4.08
T (ºC)												
DBR	33.00	35.30	36.20	34.50	34.55	35.65	35.50	37.50	36.95	36.90	37.20	33.70
DAR	33.05	35.25	37.30	34.80	36.30	36.45	36.35	36.00	35.70	34.40	38.40	35.85
LBR	34.40	36.90	38.65	39.45	40.00	40.50	41.40	42.75	43.45	45.05	41.25	41.45
LAR	34.10	35.85	37.70	38.50	39.15	38.35	39.35	40.90	40.65	41.45	39.15	39.55
TOC (mg L^{-1})	n.a.											
TSS (g L^{-1})	0.006											
NO_2^- (mg L^{-1})	<0.02											
NH_4 (mg L^{-1})	4.3											
COD (mg L^{-1})	<25											

Table S9.3. Physicochemical parameters (Dissolved oxygen (DO), pH, temperature, total suspended solids (TSS), NO_2^-, NH_4, and chemical oxygen demand (COD)) during the activated sludge simulating microcosm under dark biotic (DBR) conditions at two spiked levels of FL (10 and 100 µg L^{-1}) and no spike

	0min	30min	60min	90min	2h	3h	5h	8h	12h	24h
pH										
'No spike'	7.88	8.01	7.97	8.06	7.91	8.15	7.94	8.22	8.61	7.71
10 µg L^{-1}	7.75	7.80	7.84	7.97	7.77	8.05	8.00	8.15	8.30	8.35
100 µg L^{-1}	6.96	7.82	7.98	7.98	8.02	8.18	8.27	8.21	8.42	7.37
DO (mg L^{-1})										
'No spike'	2.01	8.75	9.11	9.15	9.3	9.50	8.51	9.35	9.15	8.71
10 µg L^{-1}	1.91	8.93	9.02	9.9	9.09	8.08	8.08	8.64	8.52	8.21
100 µg L^{-1}	1.98	8.52	8.63	8.78	8.95	8.00	8.10	8.69	8.54	8.41
T (°C)										
'No spike'	16.3	15.5	17.3	18.9	20	21.6	23.0	20.8	20.8	22.6
10 µg L^{-1}	16.2	15.9	18.0	19.1	21.4	23.1	24.3	22.3	21.6	23.7
100 µg L^{-1}	16.5	15.5	17.5	19.8	21.3	23.4	24.5	22.3	21.8	24.5
TOC (mg L^{-1})										
'No spike'	2836.0	1111.53	950.18	1078.53	1629.03	1330.47	2204.43	1655.75	902.00	1464.50
10 µg L^{-1}	---	791.49	849.03	903.38	791.55	740.18	968.97	1200.75	1231.00	1823.25
100 µg L^{-1}	---	1678.78	1022.22	1734.28	1198.03	1354.47	982.47	1354.25	7742.00	1403.75
TSS (g L^{-1})										
'No spike'	2.60									
10 µg L^{-1}	2.61									
100 µg L^{-1}	2.02									
NO_2^- (mg L^{-1})	<0.02									
NH_4 (mg L^{-1})	5.2									
COD (mg L^{-1})	103									

Table S9.4. Chromatographic parameters (retention time, R_t; resolution separation, R_s) obtained using different mobile phase modifiers (MeOH; EtOH), additives (AAC; FA) and flows.

Mobile Phase					RT (*S*/*R*)		R_s	
Organic phase (%)	Aqueuous phase (%)	Buffer concentration (mM AAC)	Acidifier (% FA)	Flow mL min^{-1}	FL	NFL	FL	NFL
80 MeOH	20	1	0.005	0.1	17.63/19.11	17.16/17.90	0.89	0.64
				0.06	35.37/38.25	34.54/35.93	1.35	0.8
80 MeOH	20	4	0.005	0.1	25.24/27.29	24.23/25.43	0.94	0.86
				0,06	50.89/55.07	48.94/50.89	1.19	0.72
80 MeOH	20	10	0.005	0.1	16.97/18.00	16.05/16.7		n.s.
				0.06	--	--	--	--
70 MeOH	30	1	0.005	0.1	15.58/16.70	14.41/15.95	1.1	0.71
				0.06	31.03/33.33	30.54/31.75	1.12	0.71
70 MeOH	30	4	0.005	0.1	24.22/25.99	23.57/24.59	0.96	0.81
				0,06	48.38/52.28	47.36/49.31	1.27	0.76
70 MeOH	30	10	0.005	0.1	16.60/17.81	16.14/16.60	--	ns
				0.06	--	--	--	--
50 EtOH	50	1		0.06	68.73/75.95	63.06/66.96	1.85	1.02
70 EtOH	30	1		0.06	78.08/86.20	71.61/76.81	1.41	1.07
80 EtOH	20	1		0.06	>90	>90		
92.5 EtOH	7.5	1		0.06	>90	>90		
70 EtOH	30	1	0.005	0.06	25.15/27.5	23.76/25.15	1.21	0.85
70 EtOH	30	4	0.005	0.06	35.37/39.55	33.79/36.12	1.41	1

Table S9.5. Performance data: linearity, R^2; instrumental and method limits of detection and quantification; resolution (R_s).

Compound	Enantiomer	Instrumental parameters					River water		Activated sludge	
		Linearity range (µg L^{-1})	R^2	$IDL_{s/n}$ (µg L^{-1})	$IQL_{s/n}$ (µg L^{-1})	R_s	MDL_{CALC} (ng L^{-1})	MQL_{CALC} (ng L^{-1})	MDL_{CALC} (ng L^{-1})	MQL_{CALC} (ng L^{-1})
						1 µg L^{-1} / 50 µg L^{-1} / 100 µg L^{-1}				
FL	*S* (+)	0.25-100	0.992	0.12	0.5	1.4 ± 0.2 / 1.4 ± 0.3 / 1.5 ± 0.4	1.2	4.6	0.8	3.1
FL	*R* (-)	0.25-100	0.991	0.12	0.5		1.3	5.1	0.5	2.0
NFL	*S* (+)	0.25-100	0.994	0.12	0.5	1.0 ± 0.4 / 1.1 ± 0.1 / 1.1 ± 0.1	1.3	5.1	0.4	1.7
NFL	*R* (-)	0.25-100	0.996	0.12	0.5		1.2	4.7	0.7	2.7

Table S9.6. Performance data: retention time, Rt; inter and intra-day repeatability.

Compound	Enantiomer	Rt (min)	Instrumental parameters: Intra-day (RSD%, n=3)									Inter-day (RSD%, n=3)			Method parameters: Intra-day (RSD%, n=3)									Inter-day (RSD%, n=3)		
			5 µg L^{-1}			25 µg L^{-1}			250 µg L^{-1}			5 µg L^{-1}	25 µg L^{-1}	250 µg L^{-1}	5 µg L^{-1}			25 µg L^{-1}			250 µg L^{-1}			5 µg L^{-1}	25 µg L^{-1}	250 µg L^{-1}
			D1	D2	D3	D1	D2	D3	D1	D2	D3				D1	D2	D3	D1	D2	D3	D1	D2	D3			
FL	S (+)	35.4	6.5	9.3	13.2	7.0	5.4	6.1	2.1	3.7	2.6	11.0	6.1	2.6	3.6	4.1	1.6	2.4	2.9	8.7	3.0	0.7	1.7	2.5	3.4	3.5
FL	R (-)	39.6	12.8	2.5	10.6	6.0	8.3	7.4	1.2	1.8	1.3	14.4	7.4	1.3	14.6	5.3	7.5	2.2	3.9	7.3	2.8	4.4	3.3	4.8	8.0	4.3
NFL	S (+)	33.8	6.5	5.4	10.1	5.5	4.4	4.2	1.0	2.1	2.0	8.4	4.2	2.0	19.8	17.0	17.6	12.2	12.3	12.2	2.3	2.2	2.1	3.2	3.9	2.7
NFL	R (-)	36.1	6.2	6.3	1.1	3.7	6.0	5.8	0.7	1.0	1.5	7.0	5.8	1.5	4.6	15.2	8.0	14.6	12.5	16.3	2.4	1.9	0.4	11.1	4.4	2.6

D1, D2, D3: days 1, 2 and 3.

Table S9.7. Transformation of FL and formation of NFL in river water simulating microcosms under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols).

	DAR					
	FL			NFL		
Time [days]	Concentration [µg L^{-1}]		EF	Concentration [µg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
1	0.478 ± 0.079	0.481 ± 0.082	0.499	n.d.	n.d.	n.d.
2	0.535 ± 0.097	0.529 ± 0.088	0.502	n.d.	n.d.	n.d.
3	0.573 ± 0.052	0.566 ± 0.061	0.504	n.d.	n.d.	n.d.
4	0.449 ± 0.036	0.437 ± 0.027	0.507	n.d.	n.d.	n.d.
5	0.479 ± 0.096	0.480 ± 0.102	0.480	n.d.	0.002 ± 0.000	0.000
6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	0.460 ± 0.079	0.443 ± 0.073	0.509	n.d.	n.d.	n.d.
9	0.459 ± 0.043	0.466 ± 0.050	0.497	n.d.	n.d.	n.d.
10	0.428 ± 0.146	0.417 ± 0.138	0.505	n.d.	n.d.	n.d.
11	0.431 ± 0.196	0.387 ± 0.174	0.526	n.d.	n.d.	n.d.
12	0.428 ± 0.142	0.420 ± 0.141	0.509	n.d.	n.d.	n.d.
13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	0.405 ± 0.077	0.386 ± 0.093	0.497	n.d.	n.d.	n.d.
16	0.470 ± 0.117	0.455 ± 0.117	0.509	n.d.	n.d.	n.d.

	DBR					
	FL			NFL		
Time [days]	Concentration [µg L^{-1}]		EF	Concentration [µg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
1	0.406 ± 0.007	0.397 ± 0.004	0.506	n.d.	0.002 ± 0.001	0.000
2	0.459 ± 0.058	0.525 ± 0.081	0.468	n.d.	n.d.	n.d.
3	0.624 ± 0.084	0.634 ± 0.081	0.496	0.001 ± 0.000	0.002 ± 0.001	0.333
4	0.497 ± 0.076	0.491 ± 0.069	0.503	n.d.	n.d.	n.d.
5	0.537 ± 0.139	0.547 ± 0.136	0.495	0.001 ± 0.000	0.002 ± 0.001	0.417
6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	0.317 ± 0.004	0.431 ± 0.197	0.441	n.d.	n.d.	n.d.
9	0.317 ± 0.055	0.319 ± 0.071	0.499	n.d.	n.d.	n.d.
10	0.299 ± 0.011	0.291 ± 0.012	0.507	n.d.	n.d.	n.d.
11	0.283 ± 0.061	0.287 ± 0.047	0.495	n.d.	n.d.	n.d.
12	0.251 ± 0.066	0.257 ± 0.035	0.490	n.d.	n.d.	n.d.
13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	0.191 ± 0.040	0.162 ± 0.026	0.538	n.d.	n.d.	n.d.
16	0.161 ± 0.057	0.127 ± 0.034	0.554	n.d.	n.d.	n.d.

Time [days]	LAR					
	FL			NFL		
	Concentration [µg L^{-1}]		EF	Concentration [µg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
1	0.485 ± 0.135	0.489 ± 0.125	0.497	n.d.	n.d.	n.d.
2	0.397 ± 0.008	0.417 ± 0.006	0.488	n.d.	0.001 ± 0.000	0.000
3	0.489 ± 0.042	0.506 ± 0.049	0.491	0.002 ± 0.001	0.004 ± 0.001	0.358
4	0.372 ± 0.047	0.377 ± 0.059	0.498	0.001 ± 0.000	0.003 ± 0.000	0.250
5	0.287 ± 0.029	0.275 ± 0.038	0.511	n.d.	0.002 ± 0.001	0.000
6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	0.275 ± 0.039	0.265 ± 0.027	0.508	n.d.	0.001 ± 0.001	0.000
9	0.227 ± 0.022	0.216 ± 0.028	0.513	n.d.	0.002 ± 0.001	0.000
10	0.219 ± 0.067	0.209 ± 0.058	0.511	n.d.	0.002 ± 0.000	0.000
11	0.243 ± 0.056	0.233 ± 0.064	0.512	n.d.	0.003 ± 0.000	0.000
12	0.187 ± 0.047	0.182 ± 0.045	0.487	0.002 ± 0.001	0.003 ± 0.001	0.225
13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	0.080 ± 0.019	0.072 ± 0.022	0.531	n.d.	0.002 ± 0.001	0.000
16	0.113 ± 0.039	0.101 ± 0.043	0.535	0.001 ± 0.000	0.004 ± 0.001	0.225

Time [days]	LBR					
	FL			NFL		
	Concentration [µg L^{-1}]		EF	Concentration [µg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
1	0.467 ± 0.059	0.481 ± 0.058	0.493	n.d.	0.001± 0.000	0.000
2	0.329 ± 0.014	0.338 ± 0.027	0.493	n.d.	0.002 ± 0.000	0.000
3	0.333 ± 0.046	0.335 ± 0.053	0.498	0.001 ± 0.000	0.002 ± 0.001	0.292
4	0.275 ± 0.098	0.273 ± 0.096	0.502	n.d.	n.d.	n.d.
5	0.278 ± 0.026	0.269 ± 0.024	0.508	0.001 ± 0.000	0.003 ± 0.001	0.292
6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
7	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	0.091 ± 0.043	0.085 ± 0.061	0.537	n.d.	n.d.	n.d.
9	0.083 ± 0.024	0.088 ± 0.059	0.512	n.d.	n.d.	n.d.
10	0.034 ± 0.019	0.043 ± 0.044	0.497	n.d.	n.d.	n.d.
11	0.051 ± 0.011	0.055 ± 0.026	0.498	n.d.	n.d.	n.d.
12	0.030 ± 0.011	0.034 ± 0.016	0.491	n.d.	n.d.	n.d.
13	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	0.013 ± 0.009	0.021 ± 0.022	0.525	n.d.	n.d.	n.d.
16	0.007 ± 0.006	0.016 ± 0.001	0.467	n.d.	n.d.	n.d.

n,a.- not analized; n.d. - not detected

Table S9.8. Transformation of FL and formation of NFL in activated sludge simulating microcosms under dark biotic (DBR) conditions at two spiked levels of FL (10 and 100 μg L^{-1}) and 'no spike'

	10 μg L^{-1} microcosm					
	FL			NFL		
Time [days]	Concentration [μg L^{-1}]		EF	Concentration [μg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
0	5.089 ± 0.305	5.490 ± 1.033	0.484	0.127 ± 0.015	0.035 ± 0.009	0.784
0.5	2.927 ± 0.216	2.879 ± 0.167	0.504	0.057 ± 0.006	0.029 ± 0.001	0.663
1	2.201 ± 0.128	4.135 ± 0.030	0.347	n.d.	0.077 ± 0.004	0.000
1.5	2.514 ± 0.002	2.768 ± 0.289	0.499	0.010 ± 0.000	0.012 ± 0.013	0.887
2	2.237 ± 0.124	2.867 ± 0.533	0.480	0.151 ± 0.068	0.050 ± 0.011	0.750
3	2.502 ± 0.251	2.875 ± 0.437	0.499	0.222 ± 0.038	0.043 ± 0.022	0.839
5	2.265 ± 0.027	3.019 ± 0.170	0.429	0.384 ± 0.053	0.070 ± 0.016	0.810
8	1.588 ± 0.529	1.811 ± 0.055	0.461	0.315 ± 0.016	0.055 ± 0.019	0.856
12	1.075 ± 0.556	2.949 ± 0.299	0.263	0.562 ± 0.219	0.126 ± 0.035	0.749
24	1.331 ± 0.000	3.001 ± 0.691	0.260	0.510 ± 0.059	0.182 ± 0.000	0.752

	100 μg L^{-1} microcosm					
	FL			NFL		
Time [days]	Concentration [μg L^{-1}]		EF	Concentration [μg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
0	49.932 ± 2.414	43.555 ± 1.748	0.531	0.553 ± 0.146	0.109 ± 0.023	0.833
0.5	49.186 ± 3.891	46.115 ± 9.344	0.519	0.538 ± 0.149	0.132 ± 0.015	0.826
1	42.865 ± 3.091	40.297 ± 2.172	0.515	0.737 ± 0.154	0.128 ± 0.038	0.858
1.5	42.894 ± 1.940	40.183 ± 0.741	0.516	0.981 ± 0.052	0.146 ± 0.039	0.872
2	47.786 ± 3.348	45.913 ± 5.113	0.511	1.890 ± 0.622	0.428 ± 0.019	0.848
3	34.498 ± 4.717	37.624 ± 3.543	0.477	2.515 ± 1.049	0.429 ± 0.164	0.852
5	26.024 ± 0.982	35.269 ± 1.956	0.431	3.535 ± 1.300	0.616 ± 0.227	0.851
8	23.630 ± 0.614	33.823 ± 4.145	0.410	3.844 ± 1.117	0.710 ± 0.193	0.843
12	22.875 ± 1.267	46.372 ± 4.755	0.326	5.346 ± 0.464	0.859 ± 0.069	0.864
24	9.842 ± 0.916	26.774 ± 0.193	0.257	5.046 ± 0.415	1.302 ± 0.066	0.794

	'No spike' microcosm					
	FL			NFL		
Time [days]	Concentration [μg L^{-1}]		EF	Concentration [μg L^{-1}]		EF
	S-FL	R-FL		S-NFL	R-NFL	
0	0.249 ± 0.003	0.241 ± 0.005	0.509	0.007 ± 0.000	0.002 ± 0.000	0.789
0.5	0.739 ± 0.008	0.638 ± 0.060	0.536	0.038 ± 0.001	0.032 ± 0.007	0.549
1	0.227 ± 0.004	0.232 ± 0.008	0.495	0.007 ± 0.001	0.002 ± 0.002	0.795
1.5	0.097 ± 0.001	0.097 ± 0.003	0.500	0.005 ± 0.001	0.001 ± 0.001	0.843
2	0.493 ± 0.014	0.623 ± 0.003	0.442	0.068 ± 0.029	0.031 ± 0.025	0.720
3	0.596 ± 0.041	0.627 ± 0.056	0.488	0.030 ± 0.004	0.007 ± 0.006	0.810
5	0.972 ± 0.043	1.403 ± 0.068	0.409	0.124 ± 0.036	0.055 ± 0.021	0.706
8	1.213 ± 0.103	2.281 ± 0.151	0.347	0.197 ± 0.008	0.028 ± 0.019	0.804
12	0.681 ± 0.091	1.072 ± 0.251	0.364	0.340 ± 0.050	0.017 ± 0.000	0.947
24	0.518 ± 0.100	0.553 ± 0.087	0.503	0.228 ± 0.049	0.043 ± 0.020	0.849

Table S9.9. Raw data of Daphtoxkit F Magna. 48 h immobile *D. magna* for each toxicant concentration.

(*R*)-FL

Concentration	Average effect	Standard deviation	Total number replicates (Rep)	N immobiles			
				Rep1	Rep2	Rep3	Rep4
0	0	0	4	0	0	0	0
0.5	0	0	4	0	0	0	0
1.5	0.25	0.5	4	1	0	0	0
5	3.25	0.5	4	3	3	4	3
16	5	0	4	5	5	5	5
50	5	0	4	5	5	5	5

(*S*)-FL

Concentration	Average effect	Standard deviation	Total number replicates (Rep)	N immobile			
				Rep1	Rep2	Rep3	Rep4
0	0	0	4	0	0	0	0
0.5	0.25	0.5	4	0	0	0	1
1.5	1	0	4	1	1	1	1
5	3.25	0.5	4	3	3	3	4
16	5	0	4	5	5	5	5
50	5	0	4	5	5	5	5

(*R*)-NFL

Concentration	Average effect	Standard deviation	Total number replicates (Rep)	N immobile			
				Rep1	Rep2	Rep3	Rep4
0	0	0	4	0	0	0	0
0.5	0.25	0.5	4	0	1	0	0
1.5	0.5	0.6	4	0	1	1	0
5	4.25	0.5	4	5	4	4	4
16	5	0	4	5	5	5	5
50	5	0	4	5	5	5	5

(*S*)-NFL

Concentration	Average effect	Standard deviation	Total number replicates (Rep)	N immobile			
				Rep1	Rep2	Rep3	Rep4
0	0	0	4	0	0	0	0
0.5	0.25	0.5	4	0	0	0	1
1.5	1	0.8	4	1	1	2	0
5	4	0.8	4	4	4	3	5
16	5	0	4	5	5	5	5
50	5	0	4	5	5	5	5

Table S9.10. *R*-FL Range

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
Control	t0	0.630	0.631	0.631	0.001	0.11%
	t24	0.223	0.265	0.244	0.030	12.17%
0.005	t0	0.650	0.644	0.647	0.004	0.66%
	t24	0.240	0.257	0.249	0.012	4.84%
0.050	t0	0.642	0.638	0.640	0.003	0.44%
	t24	0.197	0.352	0.275	0.110	39.93%
0.500	t0	0.630	0.632	0.631	0.001	0.22%
	t24	0.220	0.260	0.240	0.028	11.79%
5.000	t0	0.646	0.647	0.647	0.001	0.11%
	t24	0.402	0.427	0.415	0.018	4.26%
50.000	t0	0.646	0.647	0.647	0.001	0.11%
	t24	0.644	0.636	0.640	0.006	0.88%

Summary of Results

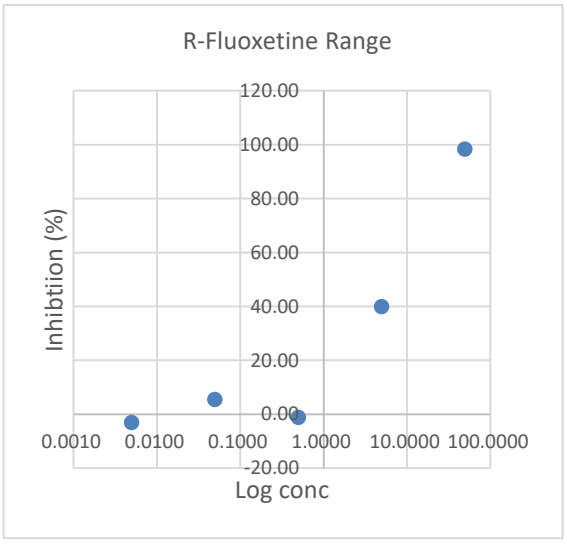
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.631	0.244
	CV%	0.11%	12.17%
0.005	Mean	0.647	0.249
	CV%	0.66%	4.84%
0.050	Mean	0.640	0.275
	CV%	0.44%	39.93%
0.500	Mean	0.631	0.240
	CV%	0.22%	11.79%
5.000	Mean	0.647	0.415
	CV%	0.11%	4.26%
50.000	Mean	0.647	0.640
	CV%	0.11%	0.88%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.244	0.387	0.000
0.005			-3.105
0.050			5.433
0.500			-1.164
5.000			39.974
50.000			98.318

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.301	-3.10	0.005
-1.301	5.43	0.050
-0.301	-1.16	0.500
0.699	39.97	5.000
1.699	98.32	50.000



Effect Concentration Results

log x =	**-1.055**
24hEC10 =	**0.088**
log x =	**-0.633**
24hEC20 =	**0.233**
log x =	**0.630**
24hEC50 =	**4.262**
log x =	**1.473**
24hEC70 =	**29.591**
log x =	**2.315**
24hEC90 =	**205.456**

Lower 95%	Upper 95%
-0.407	70.482

Summary Output

Regression Statistics	
Multiple R	0.872
R Square	0.760
Adjusted R Square	0.680
Standard Error	24.359
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5635.250	5635.250	9.497	0.054
Residual	3	1780.042	593.347		
Total	4	7415.292			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	35.037	11.138	3.146	0.051	-0.407	70.482	-0.407	70.482
X Variable 1	23.739	7.703	3.082	0.054	-0.775	48.253	-0.775	48.253

Table S9.11. *R*-FL Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
0.000	t0	0.701	0.698	0.700	0.002	0.30%
	t24	0.276	0.239	0.258	0.026	10.16%
0.029	t0	0.705	0.687	0.696	0.013	1.83%
	t24	0.293	0.260	0.277	0.023	8.44%
0.086	t0	0.701	0.698	0.700	0.002	0.30%
	t24	0.173	0.271	0.222	0.069	31.21%
2.874	t0	0.696	0.693	0.695	0.002	0.31%
	t24	0.665	0.662	0.664	0.002	0.32%
24.098	t0	0.702	0.701	0.702	0.001	0.10%
	t24	0.638	0.690	0.664	0.037	5.54%
38.556	t0	0.704	0.703	0.704	0.001	0.10%
	t24	0.696	0.646	0.671	0.035	5.27%
79.522	t0	0.697	0.709	0.703	0.008	1.21%
	t24	0.688	0.636	0.662	0.037	5.55%
120.488	t0	0.708	0.715	0.712	0.005	0.70%
	t24	0.711	0.717	0.714	0.004	0.59%

Summary of Results

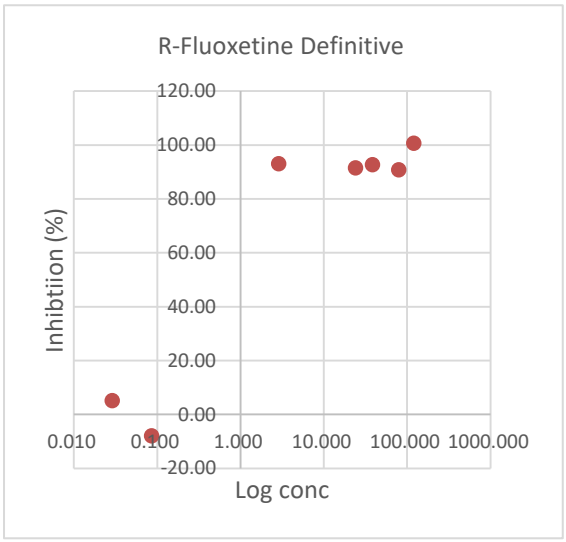
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.700	0.258
	CV%	0.30%	10.16%
0.029	Mean	0.696	0.277
	CV%	1.83%	8.44%
0.086	Mean	0.700	0.222
	CV%	0.30%	31.21%
2.874	Mean	0.695	0.664
	CV%	0.31%	0.32%
24.098	Mean	0.702	0.664
	CV%	0.10%	5.54%
38.556	Mean	0.704	0.671
	CV%	0.10%	5.27%
79.522	Mean	0.703	0.662
	CV%	1.21%	5.55%
120.488	Mean	0.712	0.714
	CV%	0.70%	0.59%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.258	0.442	0.000
0.029			5.090
0.086			-8.032
2.874			92.986
24.098			91.516
38.556			92.647
79.522			90.724
120.488			100.566

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-1.538	5.09	0.029
-1.066	-8.03	0.086
0.458	92.99	2.874
1.382	91.52	24.098
1.586	92.65	38.556
1.900	90.72	79.522
2.081	100.57	120.488



Effect Concentration Results

log x =	**-1.219**
24hEC10 =	**0.061**
log x =	**-0.882**
24hEC20 =	**0.132**
log x =	**0.130**
24hEC50 =	**1.348**
log x =	**0.804**
24hEC70 =	**6.361**
log x =	**1.479**
24hEC90 =	**30.009**

Lower 95%	Upper 95%
25.199	67.091

Summary Output

Regression Statistics	
Multiple R	0.927
R Square	0.859
Adjusted R Square	0.831
Standard Error	19.221
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	11237.216	11237.216	30.418	0.003
Residual	5	1847.140	369.428		
Total	6	13084.356			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	46.145	8.148	5.663	0.002	25.199	67.091	25.199	67.091
X Variable 1	29.653	5.377	5.515	0.003	15.832	43.475	15.832	43.475

Table S9.12. *S*-FL Range

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.681	0.671	0.676	0.007	1.05%
Control	t24	0.028	0.271	0.149	0.172	115.11%
	t0	0.653	0.668	0.661	0.011	1.61%
0.005	t24	0.204	0.302	0.253	0.069	27.39%
	t0	0.661	0.668	0.665	0.005	0.74%
0.050	t24	0.204	0.302	0.253	0.069	27.39%
	t0	0.660	0.681	0.671	0.015	2.21%
0.500	t24	0.281	0.286	0.284	0.004	1.25%
	t0	0.665	0.652	0.659	0.009	1.40%
5.000	t24	0.223	0.316	0.270	0.066	24.40%
	t0	0.662	0.669	0.666	0.005	0.74%
50.000	t24	0.431	0.454	0.443	0.016	3.68%

Summary of Results

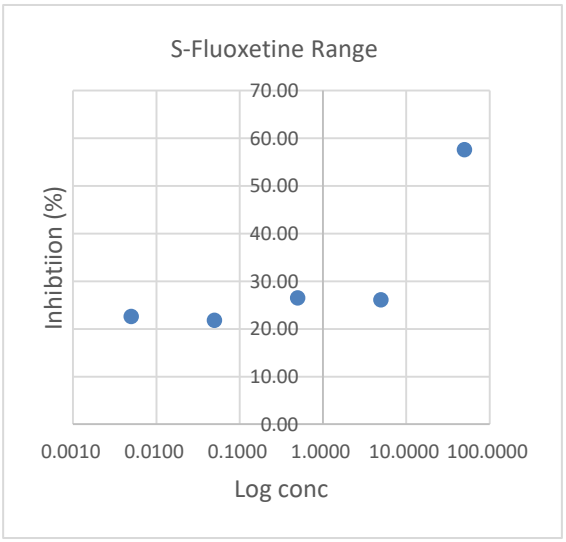
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.676	0.149
	CV%	1.05%	115.11%
0.005	Mean	0.661	0.253
	CV%	1.61%	27.39%
0.050	Mean	0.665	0.253
	CV%	0.74%	27.39%
0.500	Mean	0.671	0.284
	CV%	2.21%	1.25%
5.000	Mean	0.659	0.270
	CV%	1.40%	24.40%
50.000	Mean	0.666	0.443
	CV%	0.74%	3.68%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.149	0.527	0.000
0.005			22.617
0.050			21.857
0.500			26.510
5.000			26.130
50.000			57.653

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc
-2.301	22.62	0.005
-1.301	21.86	0.050
-0.301	26.51	0.500
0.699	26.13	5.000
1.699	57.65	50.000



Effect Concentration Results

log x =	**-3.119**
24hEC10 =	**0.001**
log x =	**-1.774**
24hEC20 =	**0.017**
log x =	**2.261**
24hEC50 =	**181.290**
log x =	**4.951**
24hEC70 =	**88210.189**
log x =	**7.641**
24hEC90 =	**42920478.224**

Lower 95%	Upper 95%
17.353	49.029

Summary Output

Regression Statistics	
Multiple R	0.780
R Square	0.609
Adjusted R Square	0.478
Standard Error	10.884
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	552.716	552.716	4.666	0.120
Residual	3	355.399	118.466		
Total	4	908.114			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	33.191	4.977	6.669	0.007	17.353	49.029	17.353	49.029
X Variable 1	7.434	3.442	2.160	0.120	-3.519	18.388	-3.519	18.388

Table S9.13. *S*-FL Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
0.000	t0	0.649	0.663	0.656	0.010	1.51%
	t24	0.174	0.208	0.191	0.024	12.59%
0.104	t0	0.641	0.643	0.642	0.001	0.22%
	t24	0.183	0.155	0.169	0.020	11.72%
0.313	t0	0.645	0.645	0.645	0.000	0.00%
	t24	0.231	0.168	0.200	0.045	22.33%
0.535	t0	0.646	0.648	0.647	0.001	0.22%
	t24	0.187	0.178	0.183	0.006	3.49%
10.446	t0	0.649	0.658	0.654	0.006	0.97%
	t24	0.189	0.255	0.222	0.047	21.02%
20.892	t0	0.645	0.649	0.647	0.003	0.44%
	t24	0.289	0.283	0.286	0.004	1.48%
68.945	t0	0.649	0.656	0.653	0.005	0.76%
	t24	0.542	0.516	0.529	0.018	3.48%
104.462	t0	0.659	0.656	0.658	0.002	0.32%
	t24	0.552	0.606	0.579	0.038	6.59%

Summary of Results

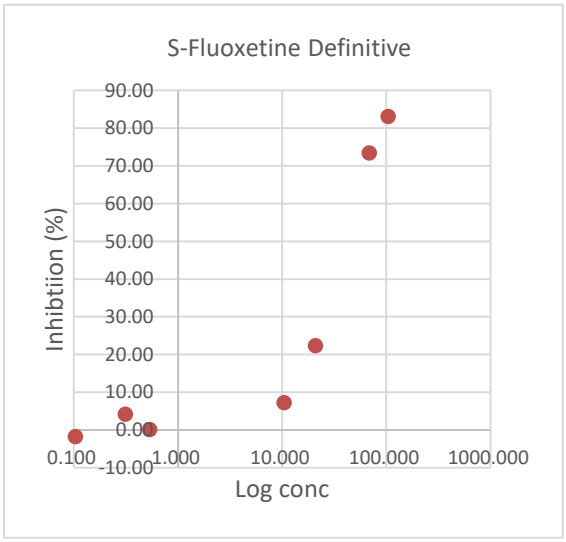
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.656	0.191
	CV%	1.51%	12.59%
0.104	Mean	0.642	0.169
	CV%	0.22%	11.72%
0.313	Mean	0.645	0.200
	CV%	0.00%	22.33%
0.535	Mean	0.647	0.183
	CV%	0.22%	3.49%
10.446	Mean	0.654	0.222
	CV%	0.97%	21.02%
20.892	Mean	0.647	0.286
	CV%	0.44%	1.48%
68.945	Mean	0.653	0.529
	CV%	0.76%	3.48%
104.462	Mean	0.658	0.579
	CV%	0.32%	6.59%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.191	0.465	0.000
0.104			-1.720
0.313			4.194
0.535			0.108
10.446			7.204
20.892			22.366
68.945			73.441
104.462			83.118

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-0.983	-1.72	0.104
-0.504	4.19	0.313
-0.272	0.11	0.535
1.019	7.20	10.446
1.320	22.37	20.892
1.839	73.44	68.945
2.019	83.12	104.462



Effect Concentration Results

log x =	**-0.039**
24hEC10 =	**0.914**
log x =	**0.358**
24hEC20 =	**2.276**
log x =	**1.549**
24hEC50 =	**35.227**
log x =	**2.343**
24hEC70 =	**218.753**
log x =	**3.137**
24hEC90 =	**1358.412**

Lower 95%	Upper 95%
-12.741	34.722

Summary Output

Regression Statistics	
Multiple R	0.843
R Square	0.710
Adjusted R Square	0.652
Standard Error	21.238
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5532.187	5532.187	12.264	0.017
Residual	5	2255.366	451.073		
Total	6	7787.553			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	10.991	9.232	1.190	0.287	-12.741	34.722	-12.741	34.722
X Variable 1	25.190	7.193	3.502	0.017	6.700	43.680	6.700	43.680

Table S9.14. *R*-NFL Range

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.655	0.655	0.655	0.000	0.00%
Control	t24	0.215	0.283	0.249	0.048	19.31%
	t0	0.646	0.646	0.646	0.000	0.00%
0.005	t24	0.372	0.194	0.283	0.126	44.48%
	t0	0.655	0.667	0.661	0.008	1.28%
0.050	t24	0.225	0.226	0.226	0.001	0.31%
	t0	0.657	0.654	0.656	0.002	0.32%
0.500	t24	0.440	0.468	0.454	0.020	4.36%
	t0	0.637	0.651	0.644	0.010	1.54%
5.000	t24	0.646	0.651	0.649	0.004	0.55%
	t0	0.693	0.675	0.684	0.013	1.86%
50.000	t24	0.716	0.708	0.712	0.006	0.79%

Summary of Results

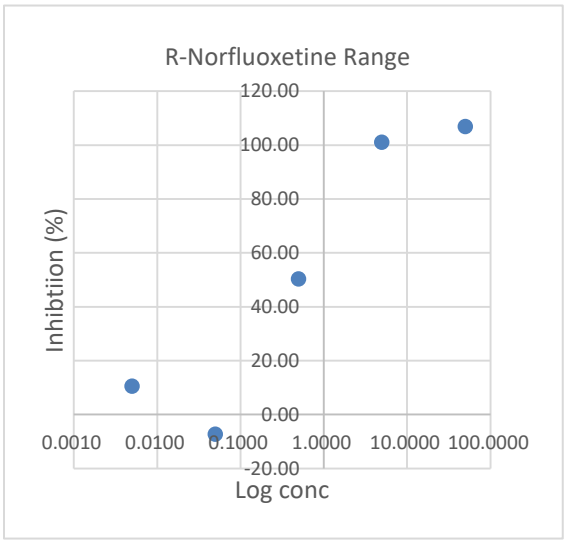
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.655	0.249
	CV%	0.00%	19.31%
0.005	Mean	0.646	0.283
	CV%	0.00%	44.48%
0.050	Mean	0.661	0.226
	CV%	1.28%	0.31%
0.500	Mean	0.656	0.454
	CV%	0.32%	4.36%
5.000	Mean	0.644	0.649
	CV%	1.54%	0.55%
50.000	Mean	0.684	0.712
	CV%	1.86%	0.79%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.249	0.406	0.000
0.005			10.591
0.050			-7.266
0.500			50.369
5.000			101.108
50.000			106.897

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.301	10.59	0.005
-1.301	-7.27	0.050
-0.301	50.37	0.500
0.699	101.11	5.000
1.699	106.90	50.000



Effect Concentration Results

log x =	**-1.708**
24hEC10 =	**0.020**
log x =	**-1.375**
24hEC20 =	**0.042**
log x =	**-0.379**
24hEC50 =	**0.418**
log x =	**0.286**
24hEC70 =	**1.929**
log x =	**0.950**
24hEC90 =	**8.895**

Lower 95%	Upper 95%
27.845	94.955

Summary Output

Regression Statistics	
Multiple R	0.922
R Square	0.850
Adjusted R Square	0.800
Standard Error	23.060
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	9059.210	9059.210	17.036	0.026
Residual	3	1595.289	531.763		
Total	4	10654.499			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	61.400	10.544	5.823	0.010	27.845	94.955	27.845	94.955
X Variable 1	30.099	7.292	4.127	0.026	6.891	53.306	6.891	53.306

Table S9.15. *R*-NFL Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.662	0.675	0.669	0.009	1.38%
0.000	t24	0.196	0.206	0.201	0.007	3.52%
	t0	0.662	0.660	0.661	0.001	0.21%
0.220	t24	0.198	0.148	0.173	0.035	20.44%
	t0	0.667	0.667	0.667	0.000	0.00%
0.661	t24	0.136	0.154	0.145	0.013	8.78%
	t0	0.668	0.686	0.677	0.013	1.88%
22.017	t24	0.635	0.653	0.644	0.013	1.98%
	t0	0.660	0.664	0.662	0.003	0.43%
35.941	t24	0.607	0.662	0.635	0.039	6.13%
	t0	0.663	0.663	0.663	0.000	0.00%
57.505	t24	0.637	0.636	0.637	0.001	0.11%
	t0	0.685	0.703	0.694	0.013	1.83%
118.604	t24	0.715	0.708	0.712	0.005	0.70%
	t0	0.698	0.699	0.699	0.001	0.10%
179.703	t24	0.718	0.721	0.720	0.002	0.29%

Summary of Results

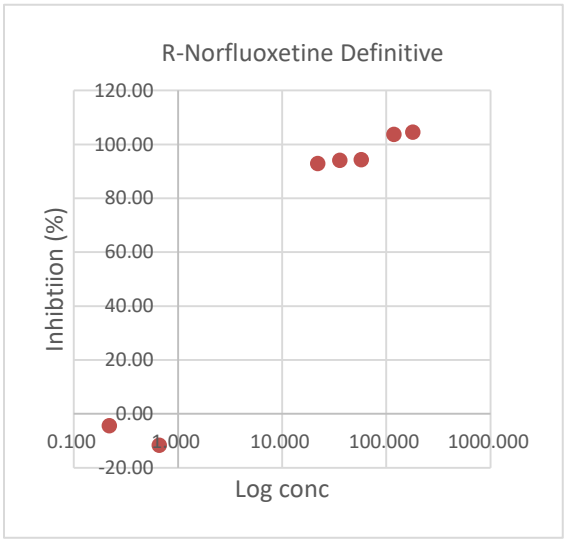
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.669	0.201
	CV%	1.38%	3.52%
0.220	Mean	0.661	0.173
	CV%	0.21%	20.44%
0.661	Mean	0.667	0.145
	CV%	0.00%	8.78%
22.017	Mean	0.677	0.644
	CV%	1.88%	1.98%
35.941	Mean	0.662	0.635
	CV%	0.43%	6.13%
57.505	Mean	0.663	0.637
	CV%	0.00%	0.11%
118.604	Mean	0.694	0.712
	CV%	1.83%	0.70%
179.703	Mean	0.699	0.720
	CV%	0.10%	0.29%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.201	0.468	0.000
0.220			-4.385
0.661			-11.658
22.017			92.941
35.941			94.118
57.505			94.332
118.604			103.743
179.703			104.492

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-0.658	-4.39	0.220
-0.180	-11.66	0.661
1.343	92.94	22.017
1.556	94.12	35.941
1.760	94.33	57.505
2.074	103.74	118.604
2.255	104.49	179.703



Effect Concentration Results

log x =	**-0.132**
24hEC10 =	**0.738**
log x =	**0.093**
24hEC20 =	**1.237**
log x =	**0.767**
24hEC50 =	**5.839**
log x =	**1.217**
24hEC70 =	**16.427**
log x =	**1.667**
24hEC90 =	**46.218**

Lower 95%	Upper 95%
-4.662	36.430

Summary Output

Regression Statistics	
Multiple R	0.969
R Square	0.938
Adjusted R Square	0.926
Standard Error	14.145
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	15190.518	15190.518	75.923	0.000
Residual	5	1000.391	200.078		
Total	6	16190.909			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	15.884	7.993	1.987	0.104	-4.662	36.430	-4.662	36.430
X Variable 1	44.469	5.104	8.713	0.000	31.350	57.588	31.350	57.588

Table S9.16. *S*-NFL Range

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.627	0.632	0.630	0.004	0.56%
Control	t24	0.251	0.184	0.218	0.047	21.78%
	t0	0.611	0.619	0.615	0.006	0.92%
0.005	t24	0.350	0.337	0.344	0.009	2.68%
	t0	1.123	0.610	0.867	0.363	41.86%
0.050	t24	0.794	0.177	0.486	0.436	89.86%
	t0	0.610	0.678	0.644	0.048	7.47%
0.500	t24	0.300	0.249	0.275	0.036	13.14%
	t0	0.619	0.622	0.621	0.002	0.34%
5.000	t24	0.380	0.364	0.372	0.011	3.04%
	t0	0.643	0.636	0.640	0.005	0.77%
50.000	t24	0.666	0.661	0.664	0.004	0.53%

Summary of Results

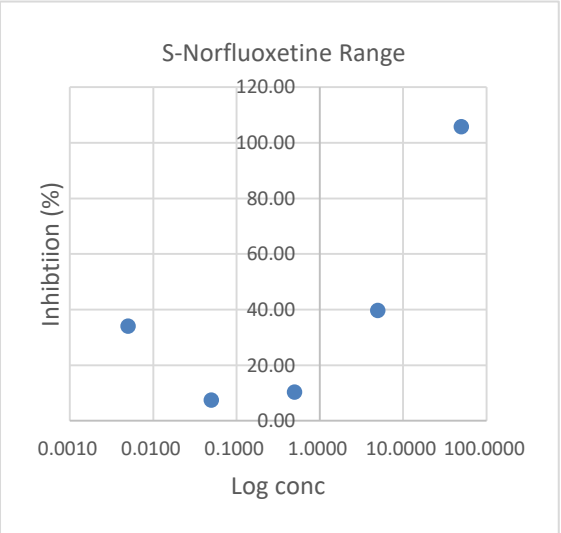
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.630	0.218
	CV%	0.56%	21.78%
0.005	Mean	0.615	0.344
	CV%	0.92%	2.68%
0.050	Mean	0.867	0.486
	CV%	41.86%	89.86%
0.500	Mean	0.644	0.275
	CV%	7.47%	13.14%
5.000	Mean	0.621	0.372
	CV%	0.34%	3.04%
50.000	Mean	0.640	0.664
	CV%	0.77%	0.53%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.218	0.412	0.000
0.005			34.102
0.050			7.524
0.500			10.316
5.000			39.684
50.000			105.825

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.301	34.10	0.005
-1.301	7.52	0.050
-0.301	10.32	0.500
0.699	39.68	5.000
1.699	105.83	50.000



Effect Concentration Results

log x =	**-1.980**
24hEC10 =	**0.011**
log x =	**-1.411**
24hEC20 =	**0.039**
log x =	**0.297**
24hEC50 =	**1.982**
log x =	**1.436**
24hEC70 =	**27.211**
log x =	**2.575**
24hEC90 =	**373.572**

Lower 95%	Upper 95%
-2.885	92.439

Summary Output

Regression Statistics	
Multiple R	0.700
R Square	0.489
Adjusted R Square	0.319
Standard Error	32.755
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3083.775	3083.775	2.874	0.189
Residual	3	3218.616	1072.872		
Total	4	6302.391			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	44.777	14.977	2.990	0.058	-2.885	92.439	-2.885	92.439
X Variable 1	17.561	10.358	1.695	0.189	-15.403	50.524	-15.403	50.524

Table S9.17. *S*-NFL Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.654	0.650	0.652	0.003	0.43%
0.000	t24	0.165	0.201	0.183	0.025	13.91%
	t0	0.641	0.637	0.639	0.003	0.44%
0.093	t24	0.206	0.203	0.205	0.002	1.04%
	t0	0.646	0.654	0.650	0.006	0.87%
0.279	t24	0.225	0.254	0.240	0.021	8.56%
	t0	0.664	0.677	0.671	0.009	1.37%
9.302	t24	0.339	0.335	0.337	0.003	0.84%
	t0	0.645	0.646	0.646	0.001	0.11%
15.352	t24	0.388	0.408	0.398	0.014	3.55%
	t0	0.659	0.661	0.660	0.001	0.21%
24.562	t24	0.566	0.568	0.567	0.001	0.25%
	t0	0.682	0.674	0.678	0.006	0.83%
50.660	t24	0.680	0.686	0.683	0.004	0.62%
	t0	0.702	0.692	0.697	0.007	1.01%
76.758	t24	0.733	0.702	0.718	0.022	3.06%

Summary of Results

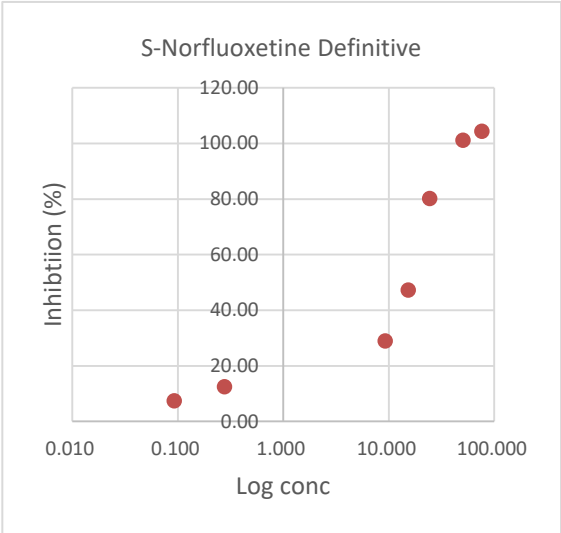
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.652	0.183
	CV%	0.43%	13.91%
0.093	Mean	0.639	0.205
	CV%	0.44%	1.04%
0.279	Mean	0.650	0.240
	CV%	0.87%	8.56%
9.302	Mean	0.671	0.337
	CV%	1.37%	0.84%
15.352	Mean	0.646	0.398
	CV%	0.11%	3.55%
24.562	Mean	0.660	0.567
	CV%	0.21%	0.25%
50.660	Mean	0.678	0.683
	CV%	0.83%	0.62%
76.758	Mean	0.697	0.718
	CV%	1.01%	3.06%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.183	0.469	0.000
0.093			7.356
0.279			12.473
9.302			28.891
15.352			47.228
24.562			80.171
50.660			101.066
76.758			104.371

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-1.032	7.36	0.093
-0.554	12.47	0.279
0.969	28.89	9.302
1.186	47.23	15.352
1.390	80.17	24.562
1.705	101.07	50.660
1.885	104.37	76.758



Effect Concentration Results

log x =	**-0.594**
24hEC10 =	**0.255**
log x =	**-0.282**
24hEC20 =	**0.523**
log x =	**0.652**
24hEC50 =	**4.483**
log x =	**1.275**
24hEC70 =	**18.785**
log x =	**1.898**
24hEC90 =	**78.719**

Lower 95%	Upper 95%
4.315	53.803

Summary Output

Regression Statistics	
Multiple R	0.891
R Square	0.794
Adjusted R Square	0.753
Standard Error	20.322
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	7947.019	7947.019	19.243	0.007
Residual	5	2064.954	412.991		
Total	6	10011.973			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	29.059	9.626	3.019	0.029	4.315	53.803	4.315	53.803
X Variable 1	32.104	7.319	4.387	0.007	13.291	50.918	13.291	50.918

Table S9.18. Non-Racemic NFL (EF=0.3) Range Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.672	0.680	0.676	0.006	0.84%
Control	t24	0.254	0.252	0.253	0.001	0.56%
	t0	0.670	0.680	0.675	0.007	1.05%
0.005	t24	0.219	0.252	0.236	0.023	9.91%
	t0	0.671	0.671	0.671	0.000	0.00%
0.050	t24	0.282	0.228	0.255	0.038	14.97%
	t0	0.688	0.675	0.682	0.009	1.35%
0.500	t24	0.221	0.266	0.244	0.032	13.07%
	t0	0.673	0.673	0.673	0.000	0.00%
5.000	t24	0.653	0.661	0.657	0.006	0.86%
	t0	0.711	0.722	0.717	0.008	1.09%
50.000	t24	0.735	0.745	0.740	0.007	0.96%

Summary of Results

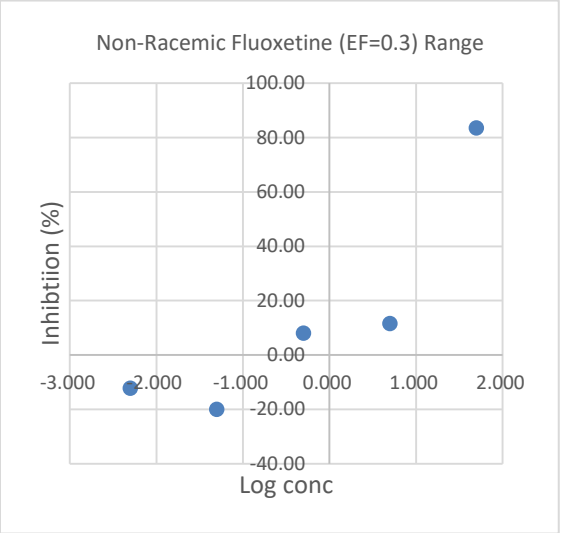
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.676	0.253
	CV%	0.84%	0.56%
0.005	Mean	0.675	0.236
	CV%	1.05%	9.91%
0.050	Mean	0.671	0.255
	CV%	0.00%	14.97%
0.500	Mean	0.682	0.244
	CV%	1.35%	13.07%
5.000	Mean	0.673	0.657
	CV%	0.00%	0.86%
50.000	Mean	0.717	0.740
	CV%	1.09%	0.96%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.253	0.423	0.000
0.005			-3.901
0.050			1.655
0.500			-3.546
5.000			96.217
50.000			105.556

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.301	-3.90	0.005
-1.301	1.65	0.050
-0.301	-3.55	0.500
0.699	96.22	5.000
1.699	105.56	50.000



Effect Concentration Results

log x =	**-1.232**
24hEC10 =	**0.059**
log x =	**-0.913**
24hEC20 =	**0.122**
log x =	**0.044**
24hEC50 =	**1.106**
log x =	**0.682**
24hEC70 =	**4.796**
log x =	**1.320**
24hEC90 =	**20.804**

Lower 95%	Upper 95%
3.221	94.045

Summary Output

Regression Statistics	
Multiple R	0.878
R Square	0.771
Adjusted R Square	0.694
Standard Error	31.209
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	9826.669	9826.669	10.089	0.050
Residual	3	2921.924	973.975		
Total	4	12748.593			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	48.633	14.270	3.408	0.042	3.221	94.045	3.221	94.045
X Variable 1	31.348	9.869	3.176	0.050	-0.060	62.755	-0.060	62.755

Table S9.19. Non-Racemic NFL (EF=0.3) Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.703	0.698	0.701	0.004	0.50%
0.000	t24	0.275	0.246	0.261	0.021	7.87%
	t0	0.726	0.706	0.716	0.014	1.98%
0.035	t24	0.321	0.264	0.293	0.040	13.78%
	t0	0.693	0.694	0.694	0.001	0.10%
0.104	t24	0.292	0.258	0.275	0.024	8.74%
	t0	0.677	0.687	0.682	0.007	1.04%
3.459	t24	0.667	0.676	0.672	0.006	0.95%
	t0	0.692	0.694	0.693	0.001	0.20%
14.679	t24	0.687	0.672	0.680	0.011	1.56%
	t0	0.690	0.695	0.693	0.004	0.51%
23.486	t24	0.675	0.680	0.678	0.004	0.52%
	t0	0.701	0.702	0.702	0.001	0.10%
48.441	t24	0.691	0.681	0.686	0.007	1.03%
	t0	0.712	0.679	0.696	0.023	3.36%
73.395	t24	0.731	0.681	0.706	0.035	5.01%

Summary of Results

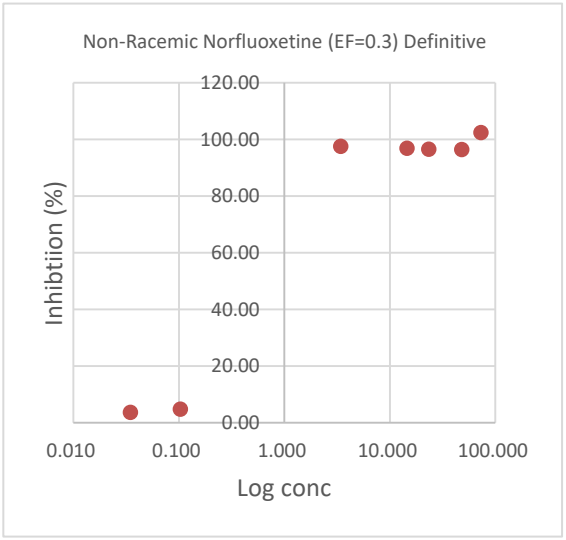
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.701	0.261
	CV%	0.50%	7.87%
0.035	Mean	0.716	0.293
	CV%	1.98%	13.78%
0.104	Mean	0.694	0.275
	CV%	0.10%	8.74%
3.459	Mean	0.682	0.672
	CV%	1.04%	0.95%
14.679	Mean	0.693	0.680
	CV%	0.20%	1.56%
23.486	Mean	0.693	0.678
	CV%	0.51%	0.52%
48.441	Mean	0.702	0.686
	CV%	0.10%	1.03%
73.395	Mean	0.696	0.706
	CV%	3.36%	5.01%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.261	0.440	0.000
0.035			3.750
0.104			4.886
3.459			97.614
14.679			96.932
23.486			96.591
48.441			96.477
73.395			102.386

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-1.456	3.75	0.035
-0.983	4.89	0.104
0.539	97.61	3.459
1.167	96.93	14.679
1.371	96.59	23.486
1.685	96.48	48.441
1.866	102.39	73.395



Effect Concentration Results

log x =	**-1.263**
24hEC10 =	**0.055**
log x =	**-0.959**
24hEC20 =	**0.110**
log x =	**-0.047**
24hEC50 =	**0.897**
log x =	**0.561**
24hEC70 =	**3.633**
log x =	**1.169**
24hEC90 =	**14.710**

Lower 95%	Upper 95%
34.251	68.857

Summary Output

Regression Statistics	
Multiple R	0.948
R Square	0.898
Adjusted R Square	0.878
Standard Error	15.991
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	11284.392	11284.392	44.127	0.001
Residual	5	1278.636	255.727		
Total	6	12563.028			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	51.554	6.731	7.659	0.001	34.251	68.857	34.251	68.857
X Variable 1	32.891	4.951	6.643	0.001	20.163	45.618	20.163	45.618

Table S9.20. Non-Racemic FL (EF = 0.3) Range

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.682	0.686	0.684	0.003	0.41%
Control	t24	0.259	0.278	0.269	0.013	5.00%
	t0	0.680	0.659	0.670	0.015	2.22%
0.005	t24	0.210	0.196	0.203	0.010	4.88%
	t0	0.689	0.668	0.679	0.015	2.19%
0.050	t24	0.189	0.170	0.180	0.013	7.48%
	t0	0.668	0.667	0.668	0.001	0.11%
0.500	t24	0.260	0.310	0.285	0.035	12.41%
	t0	0.672	0.688	0.680	0.011	1.66%
5.000	t24	0.333	0.292	0.313	0.029	9.28%
	t0	0.675	0.680	0.678	0.004	0.52%
50.000	t24	0.615	0.603	0.609	0.008	1.39%

Summary of Results

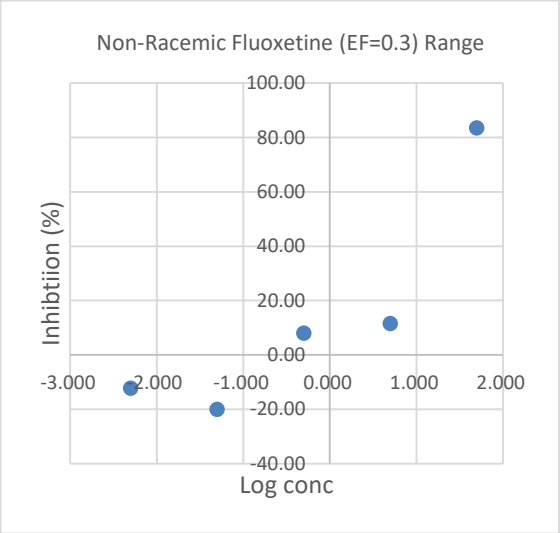
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.684	0.269
	CV%	0.41%	5.00%
0.005	Mean	0.670	0.203
	CV%	2.22%	4.88%
0.050	Mean	0.679	0.180
	CV%	2.19%	7.48%
0.500	Mean	0.668	0.285
	CV%	0.11%	12.41%
5.000	Mean	0.680	0.313
	CV%	1.66%	9.28%
50.000	Mean	0.678	0.609
	CV%	0.52%	1.39%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.269	0.416	0.000
0.005			-12.274
0.050			-20.096
0.500			7.942
5.000			11.552
50.000			83.514

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.301	-12.27	0.005
-1.301	-20.10	0.050
-0.301	7.94	0.500
0.699	11.55	5.000
1.699	83.51	50.000



Effect Concentration Results

log x =	**-0.486**
24hEC10 =	**0.327**
log x =	**-0.038**
24hEC20 =	**0.916**
log x =	**1.306**
24hEC50 =	**20.161**
log x =	**2.202**
24hEC70 =	**158.293**
log x =	**3.098**
24hEC90 =	**1242.836**

Lower 95%	Upper 95%
-14.244	55.939

Summary Output

Regression Statistics	
Multiple R	0.861
R Square	0.741
Adjusted R Square	0.654
Standard Error	24.116
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	4982.941	4982.941	8.568	0.061
Residual	3	1744.737	581.579		
Total	4	6727.678			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	20.847	11.027	1.891	0.155	-14.244	55.939	-14.244	55.939
X Variable 1	22.323	7.626	2.927	0.061	-1.947	46.592	-1.947	46.592

Table S9.21. Non-Racemic FL (EF=0.3) Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.694	0.701	0.698	0.005	0.71%
0.00	t24	0.247	0.254	0.251	0.005	1.98%
	t0	0.695	0.692	0.694	0.002	0.31%
0.012	t24	0.353	0.321	0.337	0.023	6.71%
	t0	0.681	0.678	0.680	0.002	0.31%
0.036	t24	0.324	0.313	0.319	0.008	2.44%
	t0	0.674	0.683	0.679	0.006	0.94%
1.194	t24	0.322	0.337	0.330	0.011	3.22%
	t0	0.683	0.702	0.693	0.013	1.94%
5.007	t24	0.557	0.608	0.583	0.036	6.19%
	t0	0.678	0.686	0.682	0.006	0.83%
8.011	t24	0.664	0.662	0.663	0.001	0.21%
	t0	0.674	0.672	0.673	0.001	0.21%
16.522	t24	0.669	0.669	0.669	0.000	0.00%
	t0	0.683	0.680	0.682	0.002	0.31%
25.033	t24	0.685	0.676	0.681	0.006	0.94%

Summary of Results

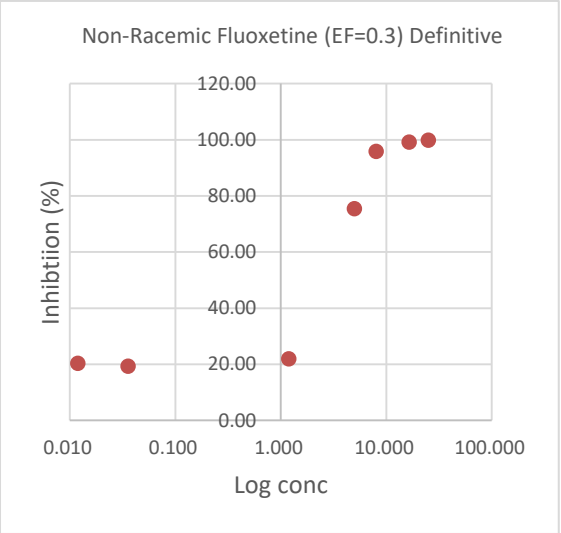
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.698	0.251
	CV%	0.71%	1.98%
0.012	Mean	0.694	0.337
	CV%	0.31%	6.71%
0.036	Mean	0.680	0.319
	CV%	0.31%	2.44%
1.194	Mean	0.679	0.330
	CV%	0.94%	3.22%
5.007	Mean	0.693	0.583
	CV%	1.94%	6.19%
8.011	Mean	0.682	0.663
	CV%	0.83%	0.21%
16.522	Mean	0.673	0.669
	CV%	0.21%	0.00%
25.033	Mean	0.682	0.681
	CV%	0.31%	0.94%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.251	0.447	0.000
0.012			20.246
0.036			19.239
1.194			21.924
5.007			75.391
8.011			95.749
16.522			99.105
25.033			99.776

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-1.923	20.25	0.012
-1.446	19.24	0.036
0.077	21.92	1.194
0.700	75.39	5.007
0.904	95.75	8.011
1.218	99.11	16.522
1.399	99.78	25.033



Effect Concentration Results

log x =	**-1.807**
24hEC10 =	**0.016**
log x =	**-1.432**
24hEC20 =	**0.037**
log x =	**-0.304**
24hEC50 =	**0.496**
log x =	**0.447**
24hEC70 =	**2.795**
log x =	**1.198**
24hEC90 =	**15.738**

Lower 95%	Upper 95%
38.957	77.252

Summary Output

Regression Statistics	
Multiple R	0.891
R Square	0.794
Adjusted R Square	0.752
Standard Error	19.592
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	7379.988	7379.988	19.227	0.007
Residual	5	1919.218	383.844		
Total	6	9299.206			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	58.105	7.449	7.801	0.001	38.957	77.252	38.957	77.252
X Variable 1	26.617	6.070	4.385	0.007	11.013	42.222	11.013	42.222

Table S9.22. Non-Racemic Mixture of FL and NFL (EF=0.3) Range

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.699	0.694	0.697	0.004	0.51%
Control	t24	0.251	0.239	0.245	0.008	3.46%
	t0	0.685	0.698	0.692	0.009	1.33%
0.005	t24	0.287	0.378	0.333	0.064	19.35%
	t0	0.690	0.686	0.688	0.003	0.41%
0.050	t24	0.299	0.393	0.346	0.066	19.21%
	t0	0.687	0.685	0.686	0.001	0.21%
0.500	t24	0.256	0.326	0.291	0.049	17.01%
	t0	0.684	0.679	0.682	0.004	0.52%
5.000	t24	0.650	0.660	0.655	0.007	1.08%
	t0	0.705	0.696	0.701	0.006	0.91%
50.000	t24	0.712	0.707	0.710	0.004	0.50%

Summary of Results

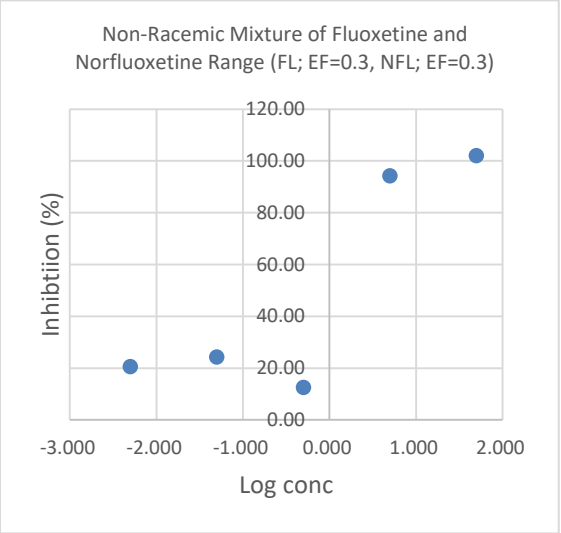
Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.697	0.245
	CV%	0.51%	3.46%
0.005	Mean	0.692	0.333
	CV%	1.33%	19.35%
0.050	Mean	0.688	0.346
	CV%	0.41%	19.21%
0.500	Mean	0.686	0.291
	CV%	0.21%	17.01%
5.000	Mean	0.682	0.655
	CV%	0.52%	1.08%
50.000	Mean	0.701	0.710
	CV%	0.91%	0.50%

Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.245	0.452	0.000
0.005			20.487
0.050			24.252
0.500			12.514
5.000			94.131
50.000			101.993

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.301	20.49	0.005
-1.301	24.25	0.050
-0.301	12.51	0.500
0.699	94.13	5.000
1.699	101.99	50.000



Effect Concentration Results

log x =	**-2.048**
24hEC10 =	**0.009**
log x =	**-1.618**
24hEC20 =	**0.024**
log x =	**-0.330**
24hEC50 =	**0.468**
log x =	**0.529**
24hEC70 =	**3.374**
log x =	**1.388**
24hEC90 =	**24.319**

Lower 95%	Upper 95%
18.606	96.766

Summary Output

Regression Statistics	
Multiple R	0.845
R Square	0.715
Adjusted R Square	0.620
Standard Error	26.857
Observations	5

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5423.792	5423.792	7.519	0.071
Residual	3	2163.899	721.300		
Total	4	7587.692			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	57.686	12.280	4.698	0.018	18.606	96.766	18.606	96.766
X Variable 1	23.289	8.493	2.742	0.071	-3.739	50.317	-3.739	50.317

Table S9.23. Non-Racemic Mixture of FL and NFL (EF=0.3) Definitive

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.679	0.688	0.684	0.006	0.93%
0.00	t24	0.227	0.202	0.215	0.018	8.24%
	t0	0.661	0.666	0.664	0.004	0.53%
0.021	t24	0.289	0.276	0.283	0.009	3.25%
	t0	0.651	0.659	0.655	0.006	0.86%
0.063	t24	0.182	0.227	0.205	0.032	15.56%
	t0	0.655	0.646	0.651	0.006	0.98%
2.091	t24	0.625	0.632	0.629	0.005	0.79%
	t0	0.662	0.678	0.670	0.011	1.69%
9.274	t24	0.657	0.636	0.647	0.015	2.30%
	t0	0.679	0.683	0.681	0.003	0.42%
14.838	t24	0.651	0.639	0.645	0.008	1.32%
	t0	0.660	0.661	0.661	0.001	0.11%
30.603	t24	0.644	0.644	0.644	0.000	0.00%
	t0	0.671	0.666	0.669	0.004	0.53%
46.369	t24	0.673	0.670	0.672	0.002	0.32%

Summary of Results

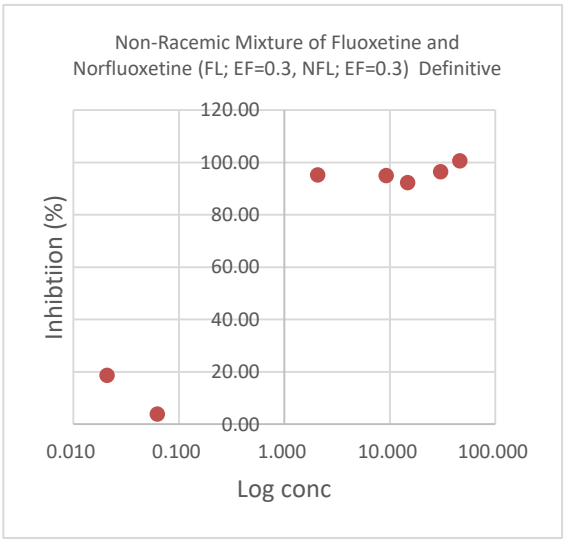
Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.684	0.215
	CV%	0.93%	8.24%
0.021	Mean	0.664	0.283
	CV%	0.53%	3.25%
0.063	Mean	0.655	0.205
	CV%	0.86%	15.56%
2.091	Mean	0.651	0.629
	CV%	0.98%	0.79%
9.274	Mean	0.670	0.647
	CV%	1.69%	2.30%
14.838	Mean	0.681	0.645
	CV%	0.42%	1.32%
30.603	Mean	0.661	0.644
	CV%	0.11%	0.00%
46.369	Mean	0.669	0.672
	CV%	0.53%	0.32%

Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.215	0.469	0.000
0.021			18.763
0.063			3.945
2.091			95.309
9.274			94.989
14.838			92.324
30.603			96.482
46.369			100.640

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-1.678	18.76	0.021
-1.201	3.94	0.063
0.320	95.31	2.091
0.967	94.99	9.274
1.171	92.32	14.838
1.486	96.48	30.603
1.666	100.64	46.369



Effect Concentration Results

log x =	**-1.724**
24hEC10 =	**0.019**
log x =	**-1.382**
24hEC20 =	**0.042**
log x =	**-0.355**
24hEC50 =	**0.442**
log x =	**0.329**
24hEC70 =	**2.134**
log x =	**1.014**
24hEC90 =	**10.300**

Lower 95%	Upper 95%
43.720	77.027

Summary Output

Regression Statistics	
Multiple R	0.933
R Square	0.871
Adjusted R Square	0.846
Standard Error	16.337
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	9035.319	9035.319	33.853	0.002
Residual	5	1334.513	266.903		
Total	6	10369.832			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	60.373	6.479	9.319	0.000	43.720	77.027	43.720	77.027
X Variable 1	29.218	5.022	5.818	0.002	16.309	42.126	16.309	42.126

PUBLICACIÓN CIENTÍFICA 10

Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety

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Analysis of cannabinoids by liquid chromatography–mass spectrometry in milk, liver and hemp seed to ensure food safety

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ABSTRACT

A method for determining cannabinoids, Δ9-tetrahidrocannabinol (THC), 11-nor-9-carboxy-Δ^9-THC (THC-COOH) and 11-hidroxy-Δ^9-THC (THC-OH) in milk, liver and hemp seeds based on liquid chromatography tandem mass spectrometry has been optimized and validated. Analytes were extracted with methanol and the extracts cleaned-up by solid-phase extraction using Oasis HLB (60 mg). The developed method was validated according to the Commission Decision 2002/657/EC. The decision limit (CCα) and detection capability (CCβ) ranged from 3.10–10.5 ng g^{-1} and 3.52–11.5 ng g^{-1}, the recoveries were 76–118% and matrix effect ranged from −17.8% to 19.9% in the three matrices studied. The method was applied to food samples obtaining positive results for THC in hemp seeds (average 0.82 μg g^{-1}) and three brands of junior formula milk at concentrations from 4.76 to 56.11 ng g^{-1}. The developed method was suitable achieving identification and quantification of cannabinoids in food matrices.

1. Introduction

Hemp (*Cannabis sativa*) is a plant able to synthetize more than 60 cannabinoids being the main active component the Δ9-tetrahidrocannabinol (THC), followed by the cannabidiol and the cannabinol (Lachenmeier, Kroener, Musshoff, & Madea, 2004). The hemp varieties allowed for cultivation in Europe have less than 0.2% THC, which is mostly present as Δ9-tetrahydrocannabinol acid (THC-A) a non-psychotropic constituent that account for 90% of total cannabinols in fiber-type cannabis plant (EFSA, 2011; Grotenhermen, 2003; Huestis, 2007; Takeda et al., 2012). However, hemp seeds have lower THC content, mainly in the external surface, as result from physical contamination with plants debris, products like hemp straw or hemp oil seed-cakes are a suitable feed material for livestock due to its high fiber content. After harvest, the THC-A begins its transformation into THC, a process quickened by heat and sunlight. Most of the acid form will be transform in THC of hemp oil seed-cakes that are obtained at high temperatures (EFSA, 2011). The noticed practice indicates that a daily amounts of 0.5 to 1.5 kg whole hemp plant dry matter can be incorporated in the daily ration of dairy cows (EFSA, 2011). The scientific opinion of the European Food Safety Authority (EFSA) on the safety of hemp (*Cannabis genus*) recommended to put whole hemp plant-derived feed materials in the list of materials banned for animal nutritional purposes and to introduce a maximum THC content of 10 mg kg^{-1} to hemp seed-derived feed material (EC., 2009; EFSA, 2011).

According to the limited number of studies performed in farm animals, after oral exposition [biodisponibility of the THC is from 6 to 30% (Ashton, 2001)], THC is metabolized in liver by oxidation through CYP 2C9 to its principal active metabolite (±)-11-hidroxy-Δ^9-THC (THC-OH) that presents higher psychotropic activity. Then, THC-OH is oxidized again to form the inactive metabolite 11-nor-9-carboxy-Δ^9-THC (THC-COOH). THC is excreted within days and weeks, mainly as metabolites, about 20–35% in urine and 65–80% in feces. Mainly the THC-COOH glucuronide is excreted in urine, and contrarily, the metabolites in the feces are only present as the non-conjugated form (Grotenhermen, 2003; Huestis, 2007). THC and its metabolites due to their lipophilic character, are distributed in the different tissues and organs, and can be excreted into milk (EFSA, 2011) as already reported in humans (Plotka, Narkowicz, Polkowska, Biziuk, & Namiesnik, 2014), squirrel monkeys (Chao et al., 1976), ruminants (Beltrán, Althaus, Molina, Berruga, & Molina, 2015), buffalos (Ahmad & Ahmad, 1990) and cows (Guidon & Zoller, 1999). In view of THC psychological effects and the EFSA concern, the quantification of the parent compounds and its metabolites in milk, liver and hemp-seeds is essential in order to ensure safe intake levels.

A summary of the analytical methods recently developed in a variety of matrices that include urine, blood, liver and milk is

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Table 10.1
Summary of different studies determining cannabinoids in several complex matrices.

Matrix	Compound	Extraction	Determination	Chromatographic time	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	Recovery (%)	References
Liver, plasma, urine and other tissues	THC, THC-OH and THC-COOH	- Previous enzymatic hydrolysis (plasma and urine) - Alkaline extraction (NaOH) + Extraction with hexane: ethyl acetate (7:1, v/v)) for THC and THC-OH - Acidic extraction (HCl) + Extraction with hexane:ethylacetate (7:1, v/v) for THC-COOH	GC–MS Derivatization (BSTFA–TMCS)	- Alkaline fraction: 20.5 min - Acidic fraction: 9.33 min	–	0.5 plasma 5 other tissues	–	Brunet et al. (2006)
Nail	THC and THC-COOH	- Alkaline hydrolysis (NaOH) + Extraction with ethyl acetate - Acidic hydrolysis (acetic acid) + Extraction with *n*-hexane: ethyl acetate (9:1, v/v)	GC–MS Derivatization (MSTFA)	16 min	*0.035–0.044	*0.2	76.3–86.6	Kim et al. (2008)
Hemp plant	THC and others cannabinoids	Methanol/chloroform (9:1, v/v)	HPLC-DAD	36 min	62.5–250	125–250	97.2–109.6	De Backer et al. (2009)
Blood	THC, THC-OH, THC-COOH and others cannabinoids	Automated on-line SPE (acetonitrile for elution)	LC–MS ESI+	10 min	0.5–3	2–8	–	Jagerdeo et al. (2009)
Hair	THC and other cannabinoids	- Alkaline hydrolysis (NaOH) + LLE with *n*-hexane: ethyl acetate (9:1, v/v) - Methanol extraction + LLE with acetonitrile	GC–MS Derivatization (MSTFA + ethyl acetate).	28.3 min	*0.02–0.05	*0.05–0.15	–	Auwarter et al. (2010)
Plasma and urine	THC, THC-OH, THC-COOH	- Plasma: Enzymatic hydrolysis and automated SPE (acetone for elution) - Urine: Enzymatic hydrolysis and automated SPE (hexane: ethyl acetate 8:2, v/v, for elution)	GC–MS Derivatization with BSTFA–TMCS	- Plasma: 31.5 min - Urine: 22.5 min	0.1	0.1 urine 0.75 plasma	61–76 (Plasma) 68–88 (Urine)	Brenneisen et al. (2010)
Milk	THC, THC-OH, THC-COOH and other illicit drugs	SPE (Methanol + dichloromethane: isopropanol 8:2, v/v with 2% ammonium hydroxide for elution)	LC–MS/MS ESI+	20 min	1.0–1.5	5.0	53.2–63.5	Marchei et al. (2011)
Urine	THC, THC-OH THC-COOH	Alkaline hydrolysis + SPE (chloroform: ethyl acetate 6:4, v/v for elution)	GC–MS Derivatization with BSTFA-1%TMCS	25 min	1.0–2.5	2.0–3	71.9–78.6	Nestic et al. (2013))
Cannabis sativa L. plant	THC and other cannabinoids	SFE (CO2 was used as extraction solvent and ethanol (20%) as co-solvent in order to modify polarity)	LC–MS/MS APCI +	28 min	0.05–2	–	–	Aizpurua-Olaizola et al. (2014)
Urine	THC, THC-OH, THC-COOH and other cannabinoids	Enzymatic hydrolysis + μ-SPE (methanol for elution)	LC–MS/MS ESI + (Except THC-COOH in ESI -)	5.8 min	2.0–4.0	6.0–10.0	65–85	Montesano et al. (2014)
Urine	Synthetic cannabinoids	Enzymatic hydrolysis + SPE (chloroform/acetone 1:1, v/v + ethyl acetate/ammonia water 94:4, v/v)	LC–MS/MS ESI+	12 min	0.1–1	0.25–1	65–99	Jang et al. (2015)

THC: Δ^9-tetrahydrocannabinol; THC-OH: (±)-11-hydroxy-Δ^9-THC; THC-COOH: 11-nor-9-carboxy-Δ^9-THC; LOD: Limit of detection; LOQ: Limit of quantification; LLE: Liquid-liquid extraction; SPE: Solid phase extraction; SFE: Supercritical fluid extraction; GC–MS: Gas chromatography-mass spectrometry; HPLC-DAD: High performance liquid chromatography with photodiode array detection; LC–MS: Liquid chromatography mass spectrometry; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; MSTFA: *N*-methyl-(trimethylsilyl) trifluoroacetamide; TMCS: trimethylchlorosilane; BSTFA: N,O-Bis (trimethylsilyl) trifluoroacetamide; ESI: electrospray ionization; APCI: atmospheric pressure chemical ionization.

* Concentration in ng mg^{-1}.

outlined in Table 10.1. Extraction and pre-concentration procedures, such as supercritical fluid extraction (SFE), solvent extraction (SE) alone or combined by solid-phase extraction (SPE) or micro-SPE (μSPE) have been applied (Auwarter, Wohlfarth, Traber, Thieme, & Weinmann, 2010; Brenneisen, Meyer, Chtioui, Saugy, & Kamber, 2010; Brunet et al., 2006; De Backer et al., 2009; Jagerdeo, Schaff, Montgomery, & LeBeau, 2009; Jang, Shin, Kim, & Yang, 2015; Kim, Cheong, Kim, Lee, & In, 2008; Marchei et al., 2011; Montesano et al., 2014; Nestic, Babic, Pavlovic, & Sutlovic, 2013). However, SE is the most widely reported. Different solvents have been used to extract these compounds depending on the matrix. Methanol/chloroform (9:1, v/v) was employed for matrices such as hemp plant and its derived products (De Backer et al., 2009; Stolker et al., 2004). Hexane/isopropanol (9:1, v/v)(Pellegrini, Marchei, Pacifici, & Pichini, 2005) combined the tissue penetration capability of the alcohol with the fat dissolution power of the hexane. For matrices as blood (Jagerdeo et al., 2009) the suit-able solvent in order to achieve the precipitation of proteins was acetonitrile. Urine and blood samples require enzymatic hydrolysis of the cannabinoids conjugates (Brenneisen et al., 2010; Jang et al., 2015; Montesano et al., 2014; Nestic et al., 2013). Table 10.1 pointed out that there are few analytical methods to determine cannabi-noids in biological matrices, rational behind them is still confusing and matrices, as milk and liver —targets to establish food safety—have been scarcely tested yet. Quantification through gas chromatography mass spectrometry (GC–MS) has been widely employed, but this procedure leads to derivatization by methylation and silylation that increases analysis time, cost and instrument maintenance (De Backer et al., 2009). Conversely, nowadays, liquid chromatography–tandem mass spectrometry (LC–MS/MS) with a triple quadrupole attains a faster cannabinoids determination without any derivatization step.

This study was aimed to develop and optimize a method to determine THC, THC-OH and THC-COOH in hemp seeds, milk and liver adapted to the current trends of green chemistry, such as reduction in the volume of solvents used and removing chlorinated solvents, thereby, achieving a more environmental friendly approach. To reach this aim, recovery tests with several SPE cartridges and elution and final extract reconstitution volumes were performed. Cannabinoids were determined by LC–MS/MS. The method was validated according to the EU qualitative control (QC) and validation guidelines Decision 2002/657/EC (EC., 2002) for cannabinoids in cow liver, milk and hemp seeds. Finally, the validated method was applied for 10 hemp seeds, 13 milk, 5 junior formula milk and 10 cow liver samples surveyed in different markets and stores of the Valencian Community (Spain). This study is one of the first preliminary attempt to determine cannabinoids in food and it just focuses on parent compound and THC biomarkers.

2. Experimental

2.1. Reagents and materials

Standard solutions of THC at 1 mg mL^{-1} and THC-COOH and THC-OH at 0.100 mg mL^{-1} in methanol were obtained from Sigma-Aldrich (The Woodlands TX, US) and LGC GMBH (Luckenwalde, Germany). THC-d3, THC-COOH-d3 and THC-OH-d3 at 0.100 mg mL^{-1} in methanol, used as isotopically labelled internal standard (IS), were also obtained from Sigma-Aldrich and LGC GMBH. The structure, molecular formula and other relevant information of the compounds are summarized in Table S10.1 in the Supplementary Material (SM). β-glucuronidase was purchased from Sigma-Aldrich.

All reagents used for sample preparation and extraction were of LC or LC/MS grade. Formic acid (94.5%) was purchased from AMRESCO (Solon, OH, US), ammonium formate (97%) from Alfa Aesar (Karlsruhe, Germany) and ammonium acetate (98%) and hydrochloric acid (HCl, 37%) from Merck KGaA (Darmstadt, Germany). Methanol was 99.9% purity and distributed for BDH Prolabo (Barcelona, Spain) and ultrapure water was produced by an Elix Milli-Q Unit (Millipore, Billarica, MA, US). Potassium phosphate buffer (pH 6.8) was prepared in the laboratory using K_2HPO_4 and KH_2PO_4 purchased from Sigma-Aldrich.

The cartridges tested for the SPE were polypropylene syringe barrels of 3 or 6 mL depending on the sorbent content and trademark. They were HyperSep C18 (200 mg) and HyperSep C8 (200 mg) from Thermo Scientific (Madrid, Spain); Supel-Select HLB (500 mg and 60 mg) Sigma-Aldrich; Strata-X 33 Reversed Phase (500 and 200 mg) from Phenomenex (Torrance, CA, US), and Oasis HLB (60 mg) from Waters (Barcelona, Spain).

2.2. Samples and sample preparation

A total of 13 milk samples (whole, semi-skimmed and skimmed), 5 junior formula (recommended for 12th month onwards and nutritionally tailored to toddler's stage of development), 10 hemp seeds and 10 liver samples were obtained from different supermarkets, fodder shops and butchers, respectively. Hemp seeds and milk were stored at 4 °C and liver samples were frozen at −20 °C until analysis.

A weight of 3 g of samples (milk, liver or hemp seeds) were aliquoted, spiked with 100 ng of each IS adding 10 μL of a mixture at a concentration of 10 μg mL^{-1} (concentration in the sample 33.3 μg g^{-1} and in the injected extract in the LC–MS/MS of 500 ng mL^{-1}). The IS was added to all samples, also to those used to determine matrix effects and recoveries, independently of whether it is used or not for calculations. Before extraction, samples were homogenized. In the case of liver, an additional step of adding 5 mL of 0.1 M potassium phosphate buffer (pH 6.8), and 200 μL β-glucuronidase solution (50,000 U/mL 0.1 M phosphate buffer) and incubated the mixture at 37 °C for 16 h was tested. As there were not recent studies on the extraction of these compounds in liver, the hydrolysis step was developed taking as starting point those studies that report this step for other substances (Croes, Goeyens, Baeyens, Van Loco, & Impens, 2009; Jedziniak, Szprengier-Juszkiewicz, Olejnik, & Żmudzki, 2010).

Then, 10 mL of methanol were added to the sample, shaken 1 min, sonicated at 40 °C for 5 min and centrifuged at 4000 rpm for 5 min. The supernatant obtained was transferred into a 100 mL volumetric flask and diluted with distilled water up to the etched ring graduation. The sample processing continued by SPE extraction, using Oasis HLB (60 mg) cartridges that were preconditioned with 2 mL of methanol, 2 mL of MilliQ water and 1 mL of a solution of ammonium acetate 100 mmol L^{-1} to activate the adsorbent and its functional groups. Then, the content of the volumetric flask (100 mL) was passed through the cartridge under vacuum. The cartridge was washed with 1 mL of HCl 0.1 mol L^{-1} to selectively remove as much compounds from the matrix as possible, while target cannabinoids remained retained on the sorbent. After the washing step, the cartridge was vacuum dried for 5 min and cannabinoids were eluted from the cartridge passing 2 mL of methanol by gravity flow. The extracts were evaporated to dryness under a gentle stream of N_2 at 30 °C, dissolved in 0.2 mL of methanol with 0.1% formic acid aided by an ultrasonic bath for 5 min and transferred to the vials for the determination by LC–MS/MS.

2.3. LC–MS/MS

LC–MS/MS was performed using an Agilent 1260 UHPLC series from Agilent Technologies (Waldbronn, Germany) equipped with

an automatic injector of 100 samples. The chromatographic separation was performed using Kinetex C18 (1.7 μm, 100 A, 50 × 2.10 mm) from Phenomenex (Torrance, CA, US) and a gradient of water with 0.1% of formic acid (phase A) and methanol with 0.1% of formic acid (phase B) at a flow rate of 0.2 mL min^{-1}. The optimum conditions were: starting at 70% of phase B increased linearly in 5 min to 95% and maintained for 7 min, then returned at 70% with an equilibration time of 12 min before the next injection. The injection volume was 5 μL.

The mass spectrometry was performed with an Agilent 6410 triple quadrupole mass spectrometer from Agilent Technologies with an electrospray ionization source working in the positive ionization (ESI^+) mode, 300 °C gas temperature, 10 L min^{-1} gas flow and 25 psi nebulizer. Acquisition was carried out in multiple reaction monitoring (MRM) mode. Two precursor ion → product ion transitions were selected per compound and fragmentor and collision energies were carefully optimized (see Table S10.2 in the SM).

2.4. Method validation

The method was validated according to the Commission Decision 2002/657/EC (EC, 2002). Linearity, recovery, decision limit (CCα), detection capability (CCβ), precision as repeatability and reproducibility, and matrix effect (ME) were studied. Linearity was established by least-squares regression analysis of the results obtained preparing a calibration curve (7-points) within the range of 10–750 μg L^{-1} for each compound (equivalent to 10–750 ng g^{-1}). The goodness-of-fit of the data were established by the determination coefficient (R^2) that indicates the proportion of the variance in the dependent variable that is predictable from the independent variable. Linearity was considered acceptable when R^2 was >0.99.

Recoveries were determined by spiking THC, THC-COOH and THC-OH at three concentration levels 50–100–500 ng g^{-1} with a final concentration of each IS of 33.3 ng g^{-1}. Each recovery level was tested in quintuplicate. These recoveries were considered acceptable in a range of 70–120%.

ME was determined using standard solutions in solvent and matrix solutions prepared by hemp seeds, milk and liver in triplicate, at seven concentrations levels into the analytical range of 10 to 750 μg L^{-1}. The ME was calculated as the ratio (in percentage) of the slopes of the standard curves prepared in matrix and in pure solvent as the following Eq. (10.1):

$$ME\ (\%) = \left(\frac{Slope\ sample}{Slope\ standard} - 1\right) \times 100 \qquad (10.1)$$

A value of 0% indicates that there is no ME. There is signal enhancement if the value is positive and signal suppression if the value is negative.

The precision of the method was determined by the repeatability and reproducibility expressed as relative standard deviation (RSDs) of 5 replicates on the same day (intra-day) and 5 different days (inter-day) at three concentration levels (50–100–500 ng g^{-1}).

In the case of substances without permitted limit established (as cannabinoids), CCα and CCβ can be established by the calibration curve procedure according to ISO 11843. For CCα blank material was used, which is fortified at around the lowest level quantifiable (the minimum required performance level is not established). Then, the signal is plot against the added concentration. CCα corresponding concentration at the y-intercept plus 2.33 times the within-laboratory standard deviation (SD). CCβ was calculated in the same way as the corresponding concentration at the CCα plus 1.64 times the SD of the within-laboratory reproducibility (β = 5%),

3. Results

3.1. LC–MS/MS method optimization

Several mixtures of water and methanol or acetonitrile, alone or with ammonium formate or formic acid were tested as mobile phases to achieve an optimal chromatographic separation as well as an optimum MS response of the cannabinoids. Both ionization modes, positive and negative, were tested pointed out that whatever the mobile phase was, the ionization in positive mode was more sensitive than in negative one. In the studies reported in Table 10.1 the determination was performed in positive ionization mode, due to the good signal intensity obtained, with the excep-tion of the study of Montesano et al. (2014) where the THC-COOH metabolite was determined in negative ionization mode.

For the mobile phase optimization, a flow of 0.2 mL min^{-1} and a concentration of cannabinoids of 100 ng mL^{-1} of standard solution were used. Of the different mixtures tested as mobile phase (shown in Figs. S10.1 and S10.2 in the SM), those with 0.1% of formic acid offered a greater sensitivity of the compounds compared to the phases with ammonium formate or solvents alone. The gradient achieved the chromatographic separation of compounds in only 10 min. The chromatographic separation obtained was short and leads to well-defined peaks. The method developed in the present study is faster than others methods reported for milk, such as the study of Marchei et al. (2011) where the retention times were 14.8 min for THC –COOH, 15.7 min for THC-OH and 17.5 min for THC. On the contrary, the study of Montesano et al. (2014) deter-mined cannabinoids in urine in 3 min, — THC-COOH at 1.68 min, THC-OH at 2.19 min and THC at 2.75 min. This separation requires a complex system of splits and valves.

The optimal quantification and confirmation transitions as well as the specific MS parameters were established for each compound (values are listed in Table S10.2 in the SM). These transitions are in agreement with those reported in studies summarized in Table 10.1. Linearity (equation and R^2) of the standards prepared in methanol as well as instrumental repeatability (intra-day-precision) and reproducibility (inter-day-precision) were at appropriate levels to apply this determination to complex food sample extract (detailed results are outlined in Table S10.3 in the SM).

This target determination only includes THC, THC-OH and THC-COOH. There are many other cannabinoids not included in this study. THC is mostly present under a precursor form (THC-A) in fresh hemp plants. Studies in both rats and humans indicate that the in vivo conversion of THC-A to THC does not occur (Zuurman, Ippel, Moin, & van Gerven, 2009). In milk samples, the presence of either THC and its metabolites as glucuronides or the excretion of THC-A is highly improbable since compounds presents in milk are apolar and slightly basic. In liver, glucuronides can be present but they can be detected including an enzymatic hydrolysis step in the extraction. Then, this chromatographic separation covers the most important metabolites.

3.2. Optimization of SPE procedure

In the present study, the SPE procedure was optimized for milk testing the type of cartridge, initial sample amount and elution and redissolution volumes. Some of these conditions are shown in Fig. 10.1. The optimized conditions were also tested and confirmed for liver samples.

Seven different cartridges, HyperSep C18 (200 mg) and C8 (200 mg), Supel-Select HLB (500 and 60 mg), Strata-X 33 Reversed Phase (500 mg and 200 mg) and Oasis HLB (60 mg), were tested. Fig. 10.1A depicts the recovery percentages obtained for each SPE car-tridge. The highest recovery percentages were provided by Oasis

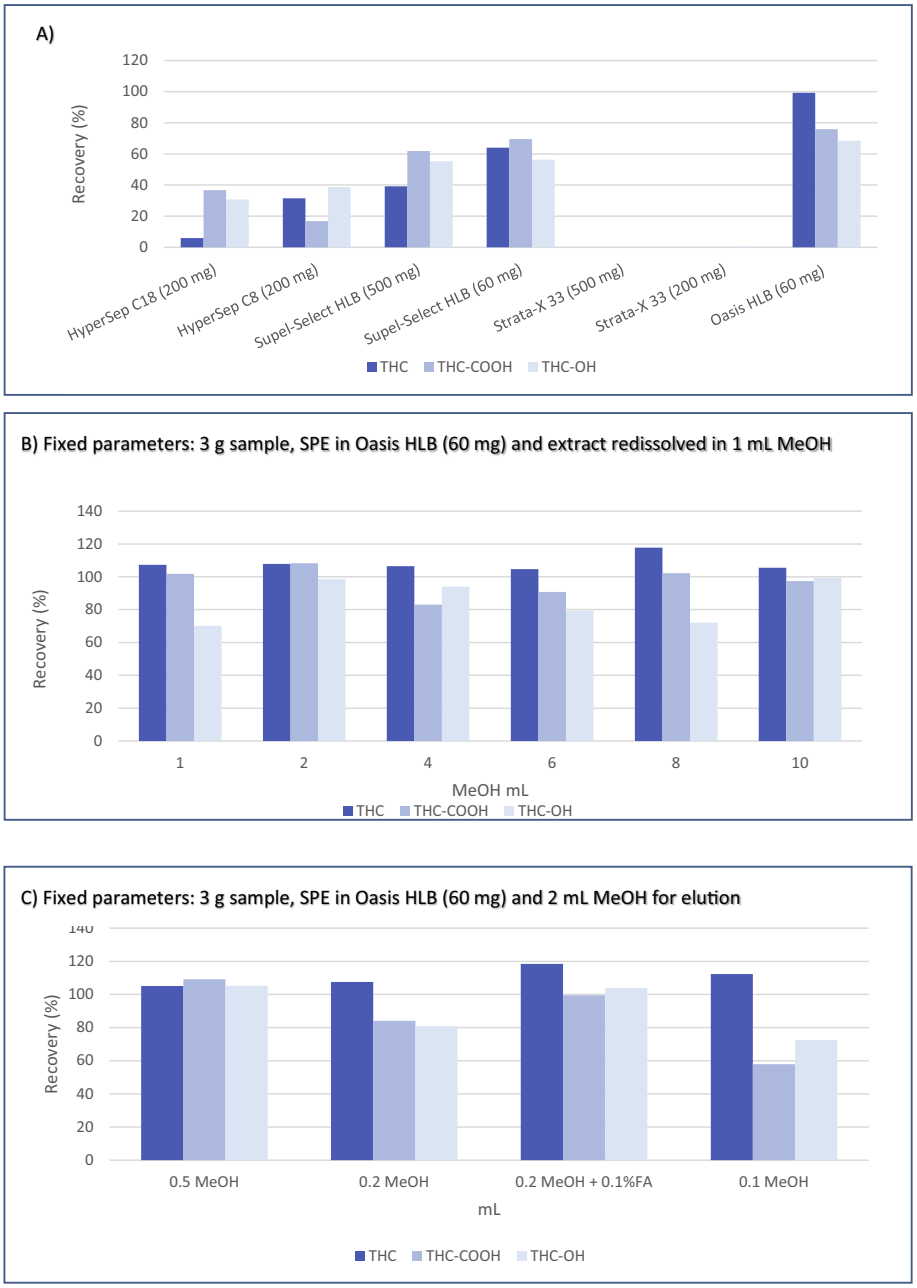

Fig. 10.1. Comparison of recoveries obtained using different (A) SPE cartridges (B) methanol elution volumes (MeOH) (C) volumes of redissolution with MeOH and MeOH + 0.1%formic acid (FA). Concentration level 500 μg L^{-1} in milk.

HLB (60 mg), ranging between 70–99%, followed by Supel-Select HLB (60 mg), which gave similar results for THC-COOH and THC-OH but lower for THC (74%). HyperSep C18 and C8 and Supel-Select HLB (500 mg) provide insufficient recoveries (<70%). Strata-X 33 Reverse Phase 500 mg and 200 mg SPE cartridge strongly retain cannabinoids, and they cannot elute with the 2 mL of methanol used as eluent. Higher methanol volumes (>8 mL) provided enough recoveries (>75%). However, the methanol volume is too high enlarging unnecessary the evaporation step and increasing the solvent consumption. Then, Oasis HLB (60 mg) was selected for further experiments. Regarding the amount of sample processed (1, 3, 5 g), the use of 5 g of sample was not possible because there is not separation of two phases after centrifugation. A probably explanation is that using 5 g of milk the proportion of methanol is not enough to precipitate the proteins. Recoveries obtained using 1 and 3 g of sample were similar (70–120%). Then, the higher amount of sample (3 g) was processed to achieve better sensitivity. The different elution volumes tested showed similar recoveries for THC, THC-COOH and THC-OH (Fig. 10.1B). The selected elution volume was the lowest volume that maintains the higher recoveries (2 mL). The same procedure was performed to select the final extract reconstitution volume (Fig. 10.1C). In this case, attending again to maintain the highest recoveries at the lowest possible volumes to achieve best sensitivity, 0.2 mL was selected as final extract reconstitution volume with recoveries ranging from 80 to 108%. The lower recoveries observed for THC-OH could be attributable to the OH negatively charged (maybe due to adsorp-tion phenomena). Then, the extract was reconstituted in methanol acidified with 0.1% of formic acid. Recoveries were better for THC-OH and more reproducible for THC-COOH.

Taking into account all studies reported in Table 10.1 that employed SPE as extraction method, the only one really comparable to the data obtained for milk is that of Marchei et al. (2011). They used methanol as eluent, but water/methanol (20:80 v/v) as redissolution solvent, obtaining recoveries within the range from 53.2 to 63.5%, lower than those achieved in this study (87–115%). The liver recoveries are not comparable with other studies shown in Table 10.1. For this matrix, an additional step of enzymatic hydrolysis was tested in order to detect glucuronides if present. This step did not modified recoveries. However, none of the liver samples present cannabinoids, its efficiency was not assessed. Clearly, in the case of spiked samples, the glucuronides do not appear because the samples were stored frozen that eliminated any residual enzy-matic activity that could remain after the sacrifice of the animals.

3.3. Validation of the analytical method

Linear calibration curves (7-points) were obtained using IS calibration in solvent and different matrices (milk, liver and hemp seeds) with R^2 of at least 0.99 in all cases (see Table S10.3 and Fig. S10.3 in the SM). Recoveries, ME, CCα and CCβ data for the three matrices obtained using IS quantification are summarized in Table 10.2. Recoveries obtained for each analyte were carried out at three concentration levels: 50, 100 and 500 ng g^{-1} in milk, liver and hemp seeds. Recoveries obtained for cannabinoids in milk ran-ged from 87 to 113% for the lowest concentration, from 95 to 115%for the medium concentration and from 92 to 114% for the highest. Recoveries obtained in liver samples were 77–96%, 95–103% and 92–117% for the lowest, medium and highest concentrations, respectively. For hemp seeds, the recoveries ranged from 76 to 118%. Comparing to other studies, Marchei et al. (2011) presented recoveries from 53.2 to 63.3% in milk and Montesano et al. (2014) reported recoveries from 73 to 81% in urine. The recovery data obtained by external calibration using the calibration curve prepared in matrix showed similar recoveries but higher RSDs (see Table S10.4 in the SM).

The ME was calculated using calibration curves in solvent (methanol), milk, liver and hemp seeds fortified at seven concentration levels from 10 to 750 μg L^{-1} using IS calibration (see Fig. S10.3 in the SM). As shown in Fig. 10.2 and Table 10.2, THC-COOH in milk (−17.8%) and THC-OH in liver (-17.3%) present signal suppression. On the contrary the MEs of THC in milk extract (7.5%) and THC-COOH in liver extract (19.9%) and hemp seeds (10.1%) show signal enhancement. This ME are low and almost negligible because normalization to the IS was used. These results also agree with those the study of Marchei et al. (2011) who reported a ME for THC ranging from −9% of suppression to 5.1% of enhancement using IS calibrations. However, the ME obtained using external cal-ibration showed always suppression in the response that averages -40% (see the detailed results in Table S10.4 in the SM).

The CCα and CCβ were, respectively, in the range of 4.13–8.73 ng g^{-1} and 4.44–8.93 ng g^{-1} for milk, 4.01–10.5 and 4.40–11.5 ng g^{-1} for liver and 3.10–6.78 and 3.52–7.22 ng g^{-1} for hemp seeds, according to the Commission Decision 2002/657/EC (EC, 2002). Values reported by Marchei et al. (2011) for milk showed a detection limit from 1 to 1.5 ng g^{-1}, and a quantification limit of 5 ng g^{-1}, which were values a little bit higher than those were obtained in this study. Again, data for liver could not be compared

Table 10.2
CCα, C C β, SPE recoveries and ME in cow milk, liver and hemp seeds ($n = 5$) obtained

Analytes		CCα (ng g^{-1})	CCβ (ng g^{-1})	SPE recoveries (%)			ME (%)
				50 ng g^{-1}	100 ng g^{-1}	500 ng g^{-1}	
THC	Milk	4.28	4.67	113 ± 18	115 ± 16	114 ± 6	7.5
	Liver	4.01	4.40	85 ± 20	103 ± 18	92 ± 8	1.4
	Hemp seeds	3.10	3.52	76 ± 12	99 ± 17	102 ± 12	2.4
THC-OH	Milk	4.13	4.44	87 ± 13	95 ± 12	113 ± 10	1.1
	Liver	8.03	8.33	77 ± 18	96 ± 15	105 ± 3	−17.3
	Hemp seeds	3.91	4.59	88 ± 13	93 ± 15	115 ± 15	−1.3
THC-COOH	Milk	8.73	8.93	93 ± 14	96 ± 15	92 ± 9	−17.8
	Liver	10.5	11.5	96 ± 14	95 ± 15	117 ± 15	19.9
	Hemp seeds	6.78	7.22	82 ± 12	90 ± 19	118 ± 12	10.1

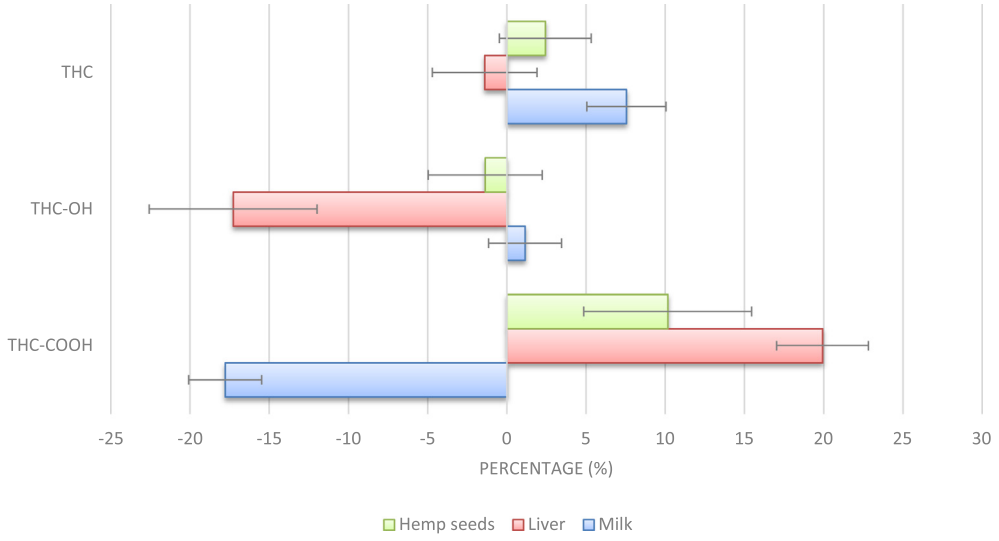

Fig. 10.2. ME obtained using IS calibration curves in a hemp seeds, milk and liver fortified at seven concentration levels from 10 to 750 μg L^{-1}.

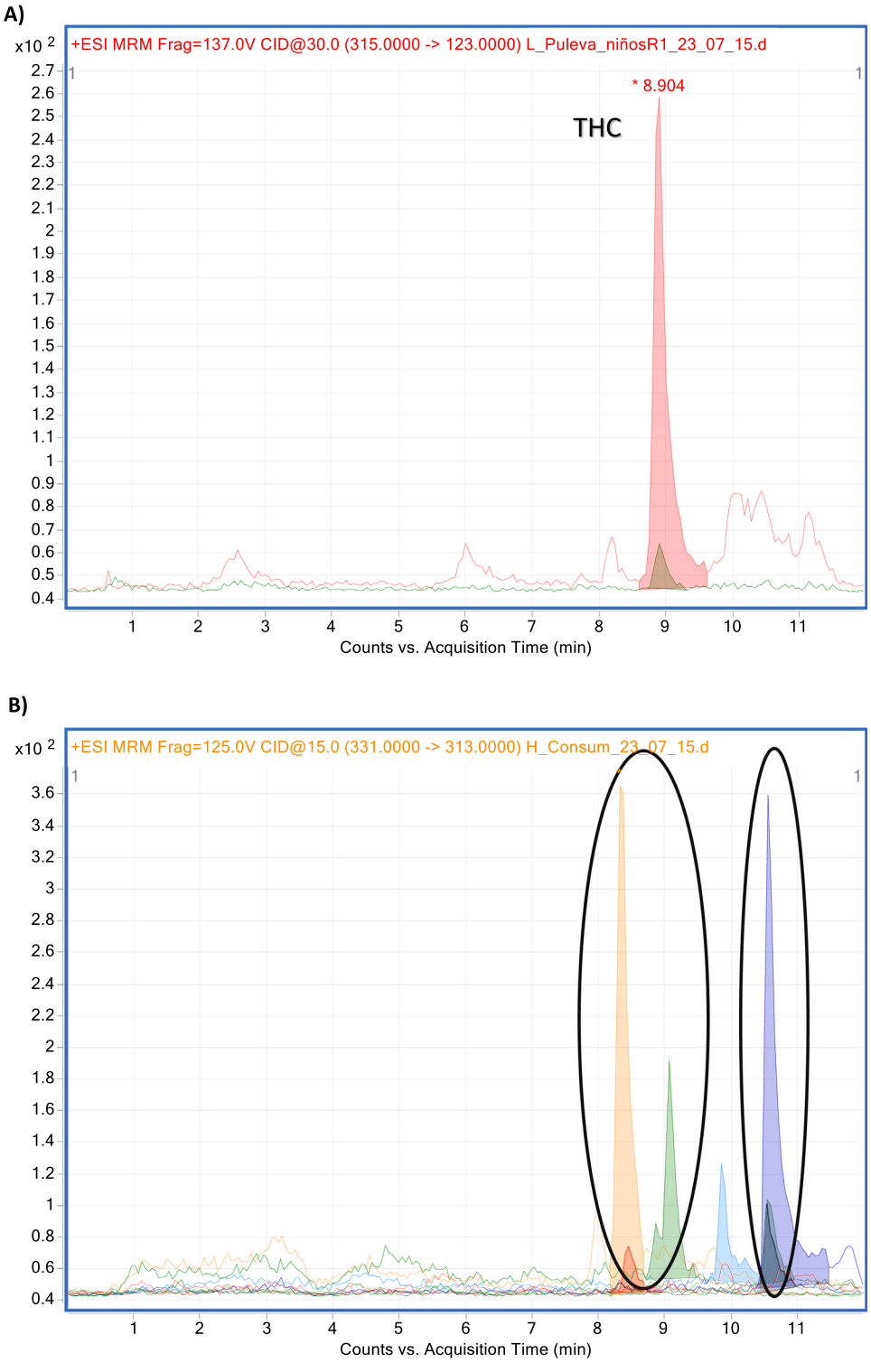

Fig. 10.3. Chromatograms of (A) positive THC in a milk sample and (B) liver sample with suspected THC metabolites.

due to the lack of studies. However, these values indicate that the proposed method has a proper sensitivity for the detections of cannabinoids in milk, liver and hemp seeds.

3.4. Analysis of samples

In milk and liver samples, neither the THC nor their metabolites were found. THC was the only compound detected in hemp seeds and in 3 of the 5 junior formulas. The detected concentration in junior formulas ranged from 4.76 ng g^{-1} to 56.11 ng g^{-1}. Recommended daily intake of milk for infants (junior formula or even milk) is two servings (400–500 mL per day) (Galiano & Moreno, 2013). So, daily intake of THC through this type of milk could be of 2.38–28.05 ng. Fig. S10.4 in the SM depicts a concentration of 0.82 mg THC kg^{-1} in hemp seeds. In the analyzed samples less than the 12 mg THC kg^{-1} established by EFSA were always detected.

Fig. 10.3 depicts the chromatograms obtained for a junior formula where THC was detected (A) and a non-contaminated liver sample (B).

Since time ago the existence of a passive exposure to THC in children due to ingestion of contaminated milk has been considered. Ahmad and Ahmad (1990) analyzed urine samples of children that drink Buffalo milk detecting low levels of THC-COOH. Astley and Little (1990) found a positive relationship between childhood exposure to THC through the breast milk and motor development within the first year of life. However, a dose-response relationship cannot be established, so their results must be interpreted with caution. Years later, Liston (1998), proved that THC could be excreted within 2 or 3 weeks after the initial exposure and feeding infants could present signals such as sedation, loss of muscle tone or bad suction. Grotenhermen (2003) showed that after oral exposure to THC through breast milk (mother smoking two marijuana cigarettes per day) the infant could ingest daily 0.01–0.1 mg THC 100 mL^{-1}.

Information on THC acute toxicity is mainly based on results obtained in animal studies, much dependent on the route of exposure and the animal concerned. THC is slightly toxic, being the data obtained for exposure by oral route, lethal dose 50 (LD_{50}) of 1270 mg kg^{-1} (male rats) and 730 mg kg^{-1} (female rats) (ISA-Scientific., 2015). (EFSA, 2011). Therefore, it is likely that, given the low THC amount found in the infant milk (approximately daily dose of 2.38–28.05 ng, depending on the brand of milk consumed) it will have little or no influence on child development. However, as young children and unborn babies are particularly sensitive, it should be studied in depth. There are studies showing that the human body is able to synthesize endocannabinoids from breast milk (compounds derived from polyunsaturated fatty acids – anandamine and 2-arachidonil glycerol) specially designed to activate cannabinoids receptors in cell membranes. The endocannabinoid system performs several key roles in neonatal and post-natal development (Garry et al., 2009). It may influence directly on the suction and infant food intake, since it interact with molecules involved in appetite regulation as leptin, ghrelin and melanocortins (Fride, Bregman, & Kirkham, 2005), learning, memory control, emotions as well as different processes to immune level. THC is an agonist for the CB_1 receptors (Garry et al., 2009) so it will act by mimicking the action of the anandamide, a neurotransmitter produced naturally by the body. Results shown that as consequence of feeding the cattle with hemp derivate food, certain amount of THC present could pass to animals being metabolized and excreted in the dairy products.

Regarding to liver processed samples, parent substance (THC) and the two metabolites (THC-OH, THC-COOH) were not detected. A published study on distribution of THC in pig tissues (Brunet et al., 2006) showed that, after an intravenous injection of 200 mg THC per kg body weight, THC is rapidly eliminated from the liver, since the concentration obtained after 0.5 h was 155 mg kg^{-1} and after 6 h was not detectable. However, Fig. 10.3B shows that in liver extract, some peaks with the same transitions as THC appear at different retention times, which could be due to the presence of other metabolites not considered in this study. THC can be conjugated given rise to other types of metabolites through their union to long-chain fatty acids, such as oleic and stearic (Grotenhermen, 2003; Huestis, 2007). More than 80 different THC conjugated metabolites that are eliminated by bile and feces (65–70%) and urine (30–35%) have been identified (Oliveró, 2000).

4. Conclusions

This study demonstrates the viability of the LC–MS/MS method for the determination of cannabinoids (THC, THC-COOH, THC-OH) in hemps seeds, milk and liver samples. The proposed method is simple, rapid and cheap providing quantitative recoveries (>70%), high reproducibility (RSDs < 20%), suitable CCαs and CCβs (4.13–8.73 μg L^{-1} and 4.44–8.97 ng g^{-1} for milk, 4.01–10.5 and 4.40–11.5 ng g^{-1} for liver and 3.10–6.78 and 3.52–7.22 ng g^{-1} for hemp seeds) and low ME (<20%). A comparison of this method with others previously reported showed clear advantages in terms of recoveries percentages obtained, time needed to determine the analytes (only required 12 min, 24 min with the post-execution).

THC was detected in hemp seeds with a concentration less than 12 mg THC kg^{-1}, the maximum limit allowed by the EU. In addition, positive results in THC were obtained for three samples of infant feeding milk at concentrations of 4.76 ng L^{-1}, 11.38 ng L^{-1} and 56.11 ng L^{-1}, respectively. In both cases, the evaluation did not identify THC metabolites or other degradation products. Considering the potential adverse effects on children development, this analytical method can be an important cornerstone not only to ensure food safety but also to gain knowledge on the hazards that cannabinoids have for human beings.

Acknowledgements

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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.foodchem.2017.01.128.

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Supplementary material

Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety.

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Table S10.1. General characteristics of the compounds analyzed in this study.

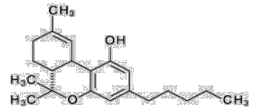
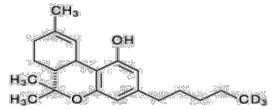
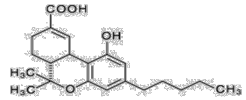
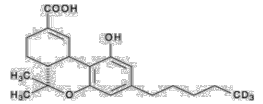
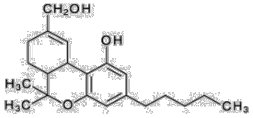
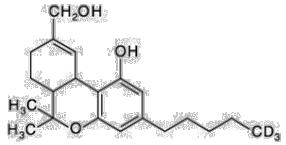
Compounds	NºCAS	Name	Chemical formula	Molecular weight (g mol^{-1})	Structure
THC	1972-08-3	Δ^9-tetrahidrocannabinol	$C_{21}H_{30}O_2$	314.47	
THC-d3	81586-39-2	(-)-Δ^9-THC-d3	$C_{21}H_{27}O_2D_3$	317.44	
THC-COOH	104874-50-2	(±)11-nor-9-carboxy- Δ^9-THC	$C_{21}H_{28}O_4$	344.44	
THC-COOH-d3	136844-96-7	(±)11-nor-9-carboxy-Δ^9-THC-d3	$C_{21}H_{25}O_4D_3$	347.46	
THC-OH	34675-49-5	(±)-11-hidroxy- Δ^9-THC	$C_{21}H_{30}O_3$	330.47	
THC-OH-d3	362044-74-4	(±)-11-hidroxy- Δ^9-THC-d3	$C_{21}H_{27}O_3D_3$	333.44	

Table S10.2. LC-MS/MS parameters for the MRM [H+] acquisition mode quantification and confirmation.

Analytes	R_T (min)	Frag (V)	MRM transitions				I (%) (RSDs %)
			Quantification (MS1)		Confirmation (MS2)		
			m/z	CE (V)	m/z	CE (V)	
THC	9.878	137	315→193	22	315→123	30	92.8 (3.6)
THC-OH	6.392	125	331→313	15	331→201	24	25.2 (11.3)
					331→193	24	20.2 (8.6)
THC-COOH	7.848	132	345→327	13	345→299	25	30.3 (2.3)
					345→193	15	42.0 (3.6)
THC-d3	8.999	108	318→196	25	318→196	22	n.d
THC-OH-d3	6.334	125	334→316	15	334→196	24	n.d
THC-COOH-d3	7.841	132	348→330	13	348→330	16	42.0 (3.6)

R_T: Retention time; Frag: Fragmentor; CE: Collision energy; MS_1: product ion for quantification; MS_2: product ion for confirmation; I: Relative intensity MS_2 compared with MS_1; RSDs: Relative standard deviation; n.d; Non detected

Table S10.3. Performance data for cannabinoids (linearity and accuracy).

	Linearity		Accuracy					
	Equation	R^2	Intra-day RSD %			Inter-day RSD %		
			50 ng g^{-1}	100 ng g^{-1}	500 ng g^{-1}	50 ng g^{-1}	100 ng g^{-1}	500 ng g^{-1}
THC	y = 0.9864x – 2.0825	0.995	4.69	0.95	1.61	8.02	1.32	3.66
THC-OH	y = 1.0585x – 6.3205	0.994	0.43	0.33	0.29	4.87	4.63	3.33
THC-COOH	y = 0.9858x – 1.9724	0.991	0.42	3.51	0.24	5.50	1.34	4.45

Table S10.4. Absolute SPE recoveries (not corrected by the IS) and ME in cow milk, liver and hemp seeds (n =3).

Analytes		**SPE recoveries (%)**			**ME (%)**
		50 ng g^{-1}	100 ng g^{-1}	500 ng g^{-1}	
THC	Milk	133 ± 22	126 ± 19	124 ± 26	-56
	Liver	67 ± 20	77 ± 18	92 ± 12	-46
	Hemp seeds	76 ± 35	89 ± 17	102 ± 14	-15
THC-OH	Milk	87 ± 17	95 ± 12	113 ± 16	-58
	Liver	77 ± 18	86 ± 15	105 ± 14	-46
	Hemp seeds	58 ± 19	83 ± 15	115 ± 25	-10
THC-COOH	Milk	63 ± 16	86 ± 15	92 ± 19	-60
	Liver	48 ± 22	85 ± 15	137 ± 28	-45
	Hemp seeds	62 ± 17	80 ± 19	128 ± 26	-20

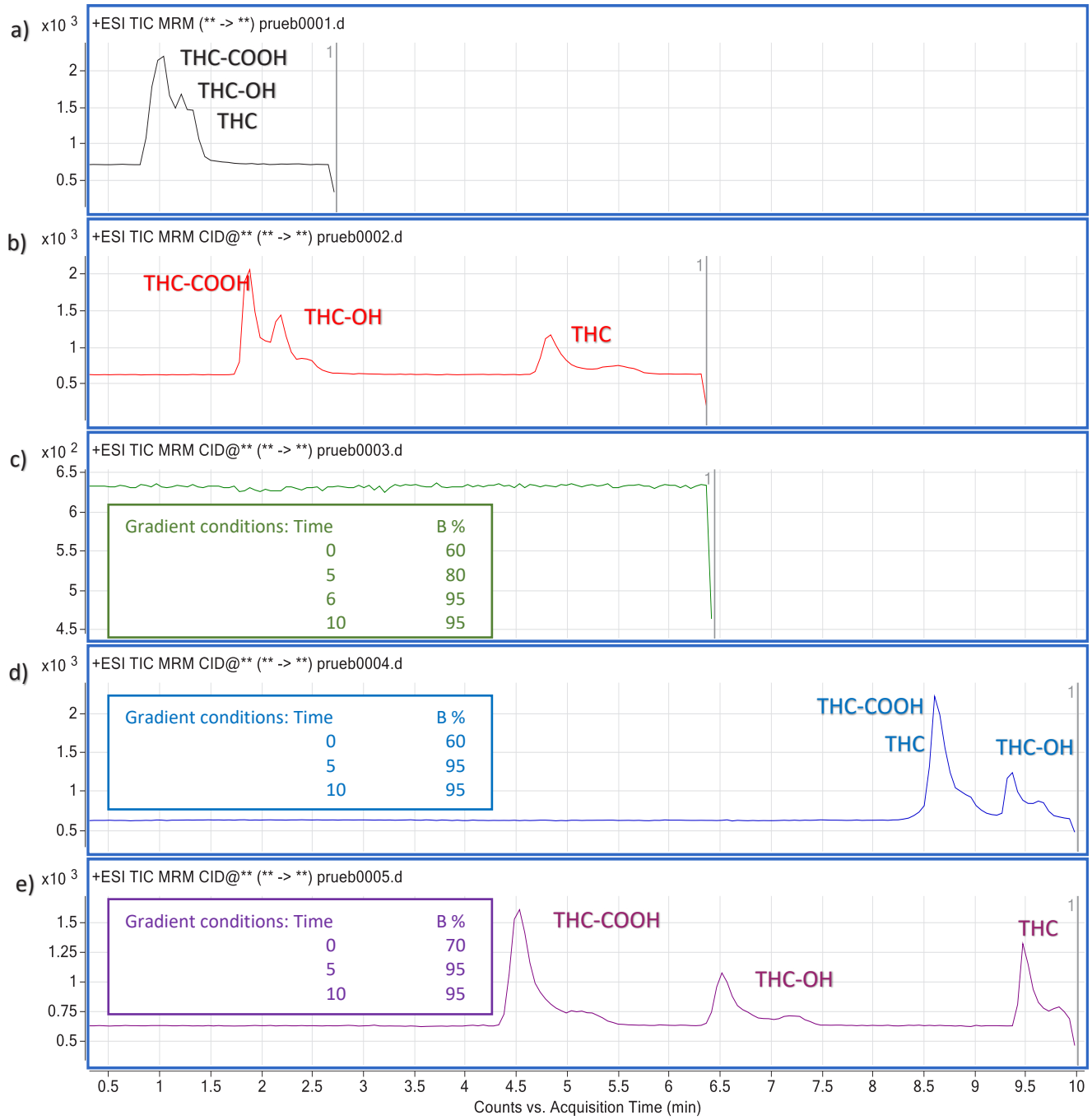


Figure S10.1. Chromatographic separation of THC, THC-COOH and THC-OH obtained for standard solution of 100 µg L^{-1}. Mobile phase composed of (A) deionized water and (B) methanol both with ammonium formate 10mM.

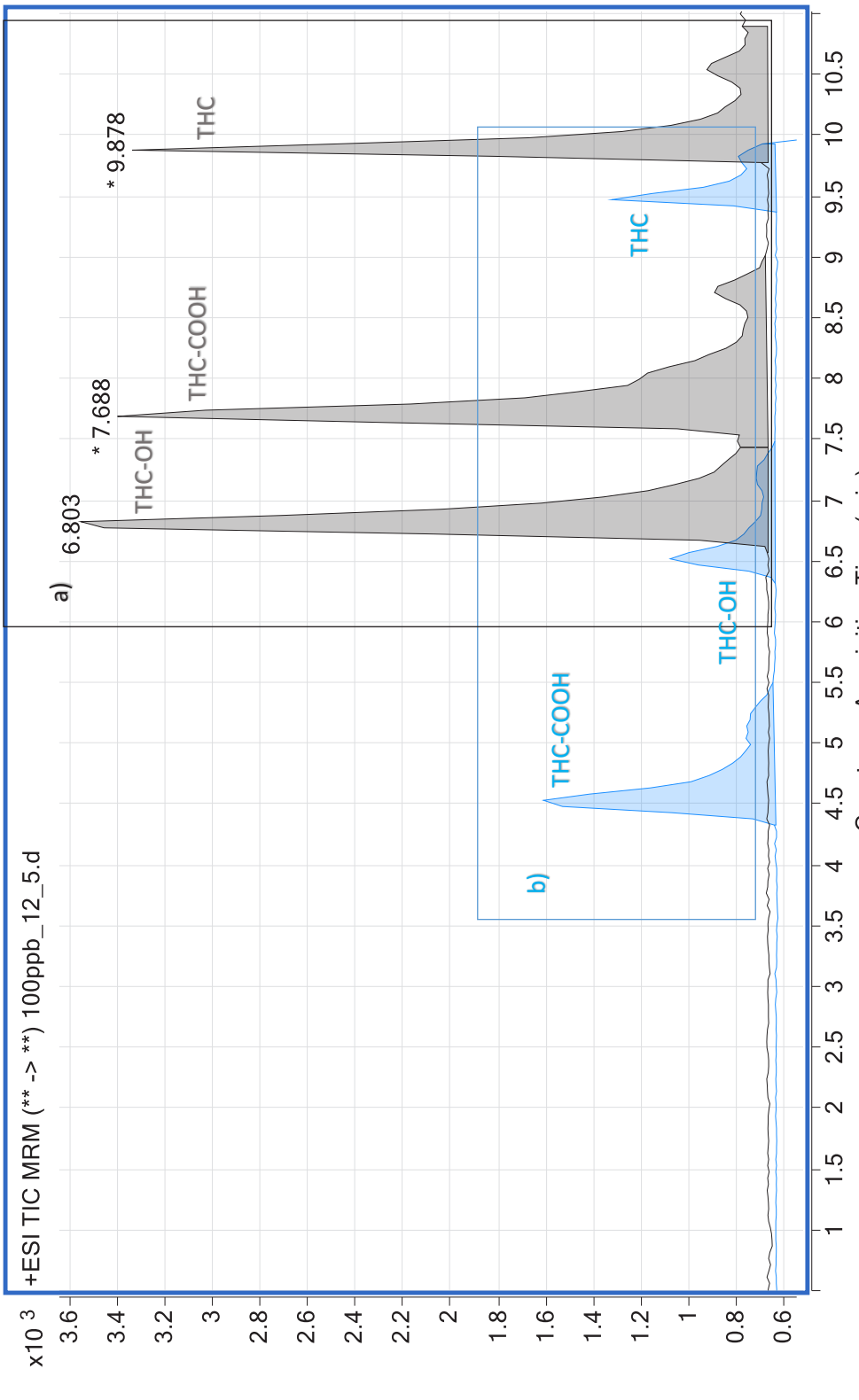


Figure S10.2. Gradient elution condition for 100 µg L^{-1} of standard compounds solution: 0 min - 70% B, 5 min - 95% B, 10 min - 95% B for a) mobile phase composed of (A) deionized water and (B) methanol both with of formic acid 0.1 % and b) (A) deionized water and (B) methanol both with ammonium formate 10mM.

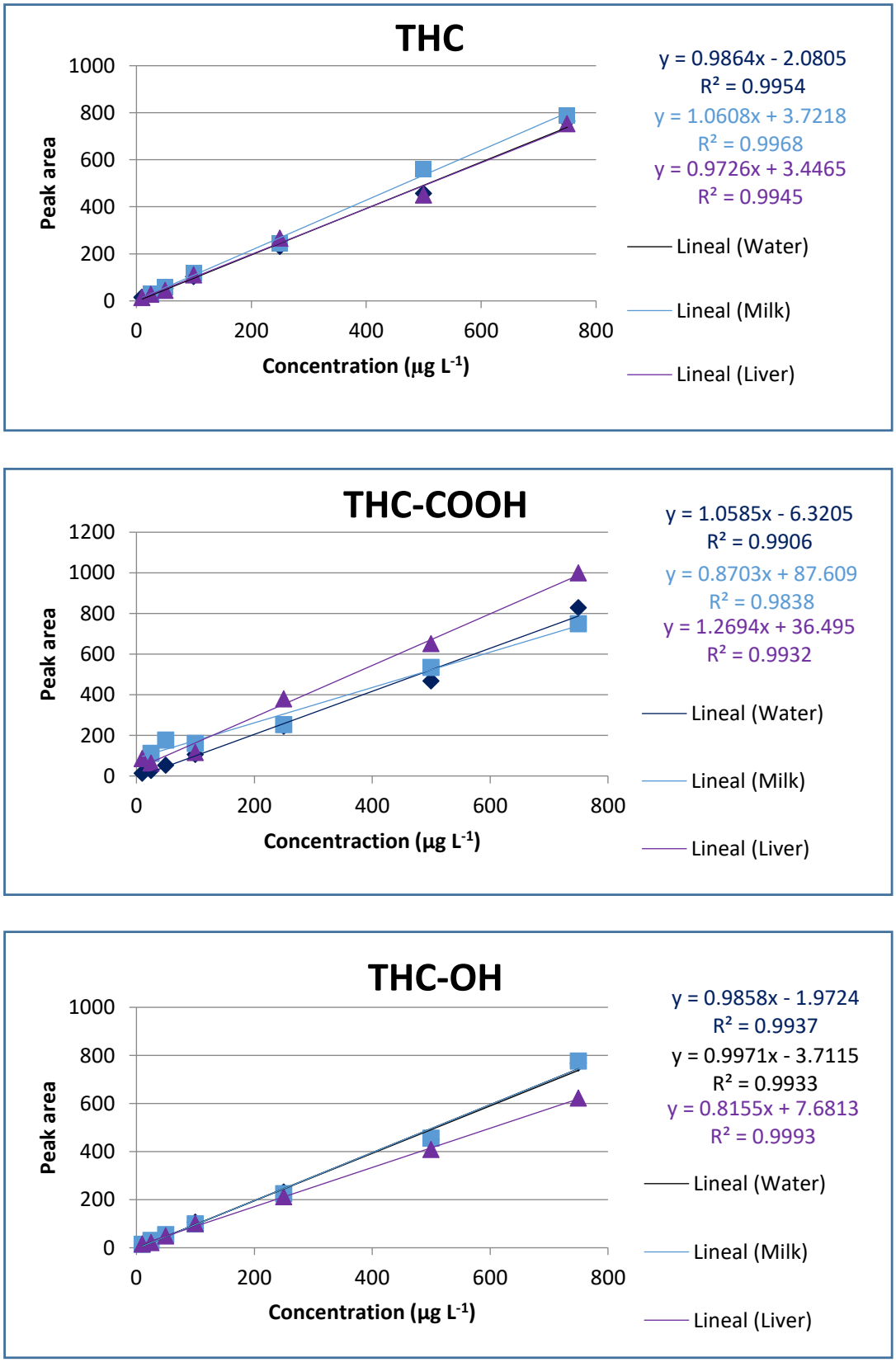


Figure S10.3. THC, THC-COOH and THC-OH linearity in complex matrices (liver and milk).

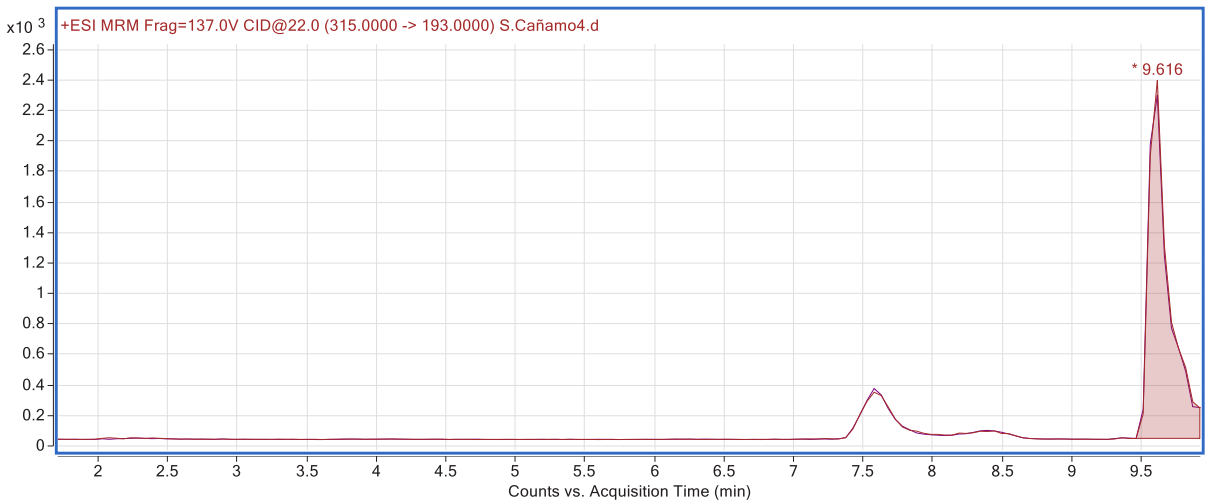


Figure S10.4. Chromatogram of THC detected in a hemp seed sample.

Capítulo 5

CAPÍTULO 5

RESUMEN

1. Desarrollo de métodos de análisis

1.1 Métodos de extracción

1.1.1 Aguas

La determinación de drogas de abuso tanto lícitas como ilícitas y sus metabolitos, fármacos, productos de higiene personal y otros bioindicadores en muestras de agua, generalmente, incluye una etapa de separación y preconcentración de los analitos. La principal dificultad inherente a esta etapa es conseguir la extracción simultánea de grupos de analitos con diferentes polaridades con una recuperación apropiada (70 – 120 %) para todos ellos.

En la presente Tesis Doctoral, este difícil reto (considerando que se han analizado más de 50 compuestos con propiedades físico-químicas muy distintas) se abordó utilizando la extracción en fase sólida (SPE) "off line". El método desarrollado se basa en la utilización de cartuchos comerciales Strata-X 33 μm (200 mg) debido a que su adsorbente polimérico en fase reversa de equilibrio hidrófilo/lipófilo (HLB), posee la capacidad de retener un amplio espectro de analitos neutros, ácidos y básicos a través de varios mecanismos que incluyen enlace π-π, puentes de hidrógeno (interacciones dipolo-dipolo) e interacción hidrofóbica.

Como paso previo a la extracción, las muestras de aguas residuales tanto de los influentes como de los efluentes, se filtran para retener los sólidos en suspensión. La filtración se realizó a vacío mediante el uso de filtros de fibra de vidrio de 90 mm de diámetro y 0.45 μm de diámetro de poro. Las aguas superficiales no se filtraron dado que la materia en suspensión era escasa.

El procedimiento de SPE consistió en acondicionar los cartuchos con 6 mL de agua desionizada y 6 mL de metanol. Seguidamente se pasaron 250 mL de las muestras de agua a través de los cartuchos Strata-X a un flujo aproximado de 10 mL min^{-1} y a continuación se dejaron secar durante 15 minutos haciendo pasar aire a su través, todo ello bajo condiciones de vacío. Los analitos retenidos en el cartucho se eluyeron con 6 mL de metanol y 3 mL de metanol-diclorometano (1:1), bajo condiciones de flujo gravitacional. Los extractos se evaporaron a sequedad mediante corriente de nitrógeno a una temperatura de 40 ºC, y el extracto se reconstituyó con 1 mL agua-metanol (9:1) para su posterior análisis por cromatografía líquida-espectrometría de masas (LC-MS).

En el estudio en el que se utilizó el cuadrupolo tiempo de vuelo (QqTOF) como analizador, se obtuvieron recuperaciones absolutas para 42 drogas de abuso y sus metabolitos, mostradas en la Tabla 3.1. Las recuperaciones se realizaron en tres matrices de aguas, influentes (42 – 92%) y efluentes (59 – 101%) de estaciones depuradoras de aguas residuales (WWTPs) y aguas superficiales (61 – 115%). Como cabía esperar, las recuperaciones obtenidas en los estudios en los que se utilizó el QqTOF como analizador fueron similares a las determinadas para el triple cuadrupolo (QqQ). En el estudio en el que se determinó la presencia de 8 drogas de abuso en aguas residuales (Tabla 5.2), las recuperaciones obtenidas para los influentes estuvieron dentro del rango de 66 – 114%. Para las aguas superficiales (Tabla S8.4) se establecieron unos rangos de recuperaciones entre 56% y 105% para las drogas de abuso analizadas.

Este método de extracción, que inicialmente se desarrolló para drogas ilícitas, se optimizó para fármacos, productos de higiene personal y cannabinoides siguiendo exactamente la misma metodología de extracción descrita anteriormente, a excepción de la proporción de agua-metanol (7:3) usada para reconstituir el extracto. Las recuperaciones absolutas obtenidas para estos compuestos se muestran en la Tabla 4.1, usando el QqTOF como analizador, en tres matrices de aguas diferentes, influentes (39 – 85%) y efluentes (52 – 101%) de WWTPs y aguas superficiales (54 – 115%).

Asimismo, este método se aplicó a la determinación biomarcadores urinarios específicos humanos. Las recuperaciones absolutas obtenidas fueron 29 – 143% (Tabla 7.2). Las recuperaciones inferiores al 70% se consideran bajas, pero hay que tener en cuenta que se tratan de recuperaciones absolutas. Estas recuperaciones podrían corregirse usando como patrones internos los patrones marcados isotópicamente con deuterio de cada uno de los analitos.

El método de extracción utilizado en la preconcentración de los enantiómeros de la fluoxetina (FL) y norfluoxetina (NFL), se basó también en la SPE, pero en este caso se utilizaron los cartuchos Oasis HLB (60 mg). Previamente, las muestras fueron filtradas como se ha explicado para las muestras de influente y efluente. El procedimiento de SPE consistió en acondicionar los cartuchos con 2 mL de agua desionizada y 2 mL de metanol. Seguidamente se pasaron 50 mL de los microcosmos de aguas superficiales o 100 mL del microcosmos de lodos activos, a través de los cartuchos a un flujo aproximado de 10 mL min^{-1} y a continuación se dejaron secar durante 30 minutos, todo ello bajo condiciones de vacío. Los analitos retenidos en el cartucho se eluyeron por gravedad con 4 mL de metanol.

Los extractos se evaporaron a sequedad en corriente de nitrógeno a una temperatura de 40 ºC, y se reconstituyeron con 0,5 mL de la fase móvil y se filtraron usando filtros de 2 μm PTFE para su posterior análisis por LC-MS. Las recuperaciones obtenidas para cada uno de los enantiómeros fueron superiores al 67% en todas las matrices estudiadas.

Además, para los biomarcadores que se estudiaron para estimar el tamaño de la población se desarrolló un método de inyección directa sin SPE basado en las técnicas de *dilute and shoot* (diluir y lanzar), que consistió en la dilución de la muestra con agua desionizada (1:1) y la eliminación de los sólidos en suspensión por centrifugación. El sobrenadante fue utilizado para su análisis por LC-MS. Este método se puso a punto, especialmente, para determinar ácido 5-hidroxiindolacético (5-HIAA) y creatinina, que no se retienen durante la SPE. En el caso de la determinación del etil sulfato también se desarrolló un método de inyección directa. A las muestras de aguas residuales se les añadió 0,5 M de tributilamina (TBA) y 0,1% de ácido fórmico (FA) como par iónico.

1.1.2 Lodos deshidratados, material particulado y sedimentos.

Se desarrolló un procedimiento para extraer las drogas de abuso tanto lícitas como ilícitas y sus metabolitos en los sólidos en suspensión de los influentes de las aguas residuales, lodos deshidratados procedentes de los tratamientos de las WWTPs y sedimentos depositados por las aguas superficiales en los ríos. Este procedimiento se basó en una extracción sólido-líquido (SLE) con disolventes, seguida de una purificación del extracto utilizando la SPE "off line". Los filtros con el material particulado se cortaron en dos mitades, una de ellas (1 - 2,5 g) se utilizó para la extracción de los analitos, así como 1 g de sedimento o lodos deshidratados. La muestra se sometió a una extracción asistida por ultrasonidos (UAE) con 10 ml de tampón McIlvain-metanol pH 4.5 (1:1, v/v). Seguidamente se homogeneizaron, sonicaron y centrifugaron. El sobrenadante se trasvasó a un matraz aforado de 250 ml, se ajustó a pH 6 con NaOH 1 M y se enrasó con agua destilada. Posteriormente se realizó la extracción de los analitos utilizando la SPE detallada en el apartado 1.1.1.

Las recuperaciones obtenidas en la puesta a punto del método de extracción se detallan en la Tabla 2.3. Este método permitió obtener recuperaciones entre 62% y 125% para todos los analitos, a excepción de tres de ellos (efedrona (EPHED) 31%, ecgonine methyl ester (ECME) 40% y 4-acetoxy-N,N-dimetiltriptamina (4-AcO-DIPT) 45%). Las recuperaciones relativas obtenidas para cada una de las matrices se detallan en la Tabla 2.5.

1.1.3 Alimentos

Se desarrolló una metodología de extracción para determinar cannabinoides en semillas de cáñamo, leche (entera, semidesnatada y desnatada), fórmulas de crecimiento e hígado de vaca.

Para la preparación de la muestra se homogeneizaron 3 g de semillas de cáñamo, leche o hígado. En el caso del hígado, se realizó una etapa adicional que consistió en la adición de 5 ml de tampón de fosfato potásico (0,1 M, pH 6,8) y 200 µl de solución de β-glucuronidasa (50.000 U/mL 0,1 M de tampón de fosfato) y se incubó la mezcla a 37 °C durante 16 horas. A continuación, se añadieron 10 ml de metanol a la muestra, se agitaron, se sonicaron y se centrifugaron. El sobrenadante obtenido se transfirió a un matraz aforado de 100 ml y se enrasó con agua.

Posteriormente se realizó una purificación de los extractos mediante la SPE "off line". Se probaron 7 cartuchos diferentes (HyperSep C18 (200 mg) y C8 (200 mg), Supel-select HLB (500 mg y 60 mg), Strata-X 33 µm (500 mg y 200 mg) y Oasis HLB (60 mg)). Se optó por los cartuchos Oasis HLB (60 mg), los cuales proporcionaron las mejores recuperaciones. Los cartuchos se acondicionaron con 2 mL de agua desionizada, 2 mL de metanol y 1 mL de la solución de acetato de amonio 100 mmol L^{-1}. Seguidamente se pasaron 100 mL del extracto a través de los cartuchos HLB a un flujo aproximado de 10 mL min^{-1}, se lavaron con 1 mL de HCl 0,1 mol L^{-1} y a continuación se dejaron secar durante 5 minutos, todo ello bajo condiciones de vacío. Los analitos retenidos en el cartucho se eluyeron a gravedad con 2 mL de metanol. Los extractos se evaporaron a sequedad mediante corriente de nitrógeno a una temperatura de 30 ºC, y los analitos se reconstituyeron con 0,2 mL metanol con 0,1% de FA para su posterior análisis en el LC-MS. En el proceso de optimización del método de extracción para estas matrices, además de distintos cartuchos de extracción, se ensayaron distintos volúmenes de elución y reconstitución del extracto. Las recuperaciones se llevaron a cabo a tres niveles de concentración, obteniéndose valores entre 87 – 115% para leche, 77 – 117% para hígados y 76 – 118% para semillas de cáñamo (los valores detallados se pueden consultar en la Tabla 10.2).

1.2. Métodos de determinación

1.2.1 Cromatografía líquida-espectrometría de masas con analizador de triple cuadrupolo (QqQ).

La separación cromatográfica se llevó a cabo utilizando una columna analítica Kinetex C_{18} (1,7 μm, 100A, 50 x 2,10 mm) y una fase móvil compuesta por metanol-agua, ambos con un 0,1% de FA para todos los compuestos analizados, a excepción de los enantiómeros de la FL y la NFL. En estos se utilizó una columna quiral Astec Chirobiotic V, CBV (5 μm, 25 cm x 2,1 mm) y una fase móvil compuesta por metanol-agua 4 mM de acetato de amonio y 0,005% de FA.

La detección se realizó mediante un analizador QqQ en modo de monitorización de reacciones múltiples (MRM) o monitorización de reacciones seleccionadas (SRM) (transiciones ion precursor→ion producto). Se seleccionaron dos transiciones por compuesto para cumplir los requerimientos establecidos por la UE para confirmar la identidad de los analitos (Decisión de la Comisión 2002/657/CE). Las condiciones de las dos transiciones de cada uno de los compuestos estudiados fueron optimizadas con el fin de obtener la máxima sensibilidad mediante la selección de los parámetros óptimos para cada ion producto (fragmentador y energía de colisión). La transición más abundante se usó con fines cuantitativos y la menos abundante con fines cualitativos. Las Tablas 2.2, S5.1, S7.5, S8.3 y S10.2 listan las transiciones utilizadas y los tiempos de retención de cada uno de los analitos. Las transiciones para el etil sulfato se presentan en el artículo científico 6 y las de la FL y NFL en el artículo científico 9.

La validación del método se llevó a cabo para cada matriz y analitos estudiados. Los límites de detección (LODs) y los límites de cuantificación (LOQs) se muestran en las Tablas 2.4, 5.2, 7.2, S8.4, S9.5 y 10.2. Los LODs y LOQs se determinaron experimentalmente como la concentración del analito que proporcionó una señal-ruido de 3:1 y 10:1, respectivamente. La sensibilidad de los compuestos seleccionados fue satisfactoria para todas las matrices estudiadas desde el punto de vista medioambiental, al menos hasta que los estudios sobre los efectos toxicológicos o sobre la salud humana no indiquen la necesidad de determinarlos a niveles inferiores.

La precisión expresada como la desviación estándar relativa (%RSD) intra- e inter-día, en todos los casos fue ± 20%. Las curvas de calibración presentaron unos coeficientes de correlación (r^2) superiores a 0,99.

Los efectos de matriz para los compuestos estudiados se calcularon obteniendo una supresión o aumento de su señal. En todos los casos se utilizó un patrón interno (el analito marcado isotópicamente con deuterio) para determinar su concentración. En los casos en los que no se dispuso del patrón deuterado del propio analito se utilizó aquel más próximo

en cuanto a estructura química y tiempo de retención al analito a determinar. Los patrones internos se añadieron a las muestras en el inicio de su procesamiento y antes del proceso de extracción, por lo que el efecto matriz quedó corregido.

1.2.2. Cromatografía líquida-espectrometría de masas con analizador de cuadrupolo tiempo de vuelo (QqTOF).

Las muestras de aguas superficiales y aguas residuales, se analizaron utilizando UHPLC-QqTOF-MS, con el fin de determinar cuantitativamente los compuestos objetivo (*target*), así como de realizar identificaciones cualitativas "no dirigidas" de metabolitos y productos de degradación de dichos compuestos y de supuestos contaminantes emergentes.

La separación cromatográfica se llevó a cabo utilizando una columna analítica C_{18} y una fase móvil compuesta por metanol-agua, ambas con un 0,1% de FA para los compuestos de ionización en positivo y una fase móvil metanol-agua, ambas con 2,5 mM de fluoruro de amonio para los compuestos de ionización en negativo.

La identificación de todos los compuestos se llevó a cabo mediante un analizador QqTOF-MS/MS. El espectro de masas de las drogas de abuso se obtuvo usando una fuente de electrospray en modo de ionización positiva (Tabla S3.2) y los fármacos, productos de higiene personal y cannabinoides en modo de ionización negativo (Tabla 4.3). En ambos casos el rango de los espectros de masas osciló entre 100 – 700 m/z con una fuente Turbo Ionspray. Para el análisis cualitativo "no dirigido" se utilizaron ambas ionizaciones. Los espectros MS/MS de los iones producto se obtuvieron en el modo de adquisición dependiente de la información (IDA). En la Figura 3.1 se puede observar el cromatograma de los iones totales (TIC) para los experimentos MS e IDA-MS/MS, en una muestra de agua destilada adicionada con los patrones de las drogas de abuso ionizadas en positivo seleccionadas para el estudio.

Las tablas 3.1 y 4.1 muestran los parámetros obtenidos en la validación de los métodos aplicados para agua residual (influentes y efluentes) y superficial. La sensibilidad del método se estableció mediante el nivel más bajo de calibración (LCL o LOQ) en el que la concentración de cada uno de los analitos estudiados diera un recuento de intensidad mayor a $1{,}0 \times 10^4$. Los LCL o LOQ oscilaron en el rango 1 -150 ng L^{-1}. Generalmente, los LCL o LOQ fueron más bajos en la matriz con menor materia residual, como las aguas superficiales, y mayores en matrices como los influentes de aguas residuales.

Las Tablas y Figuras S3. se pueden encontrar en el Supporting Information – A en http://dx.doi.org/10.1016/j.chroma.2016.07.062

La precisión (%RSD) intra- e inter-día, en todos los casos fue de ± 20%. Las curvas de calibración presentaron unos r^2 superiores a 0,99, excepto para 4-AcO-DIPT, heroína (HER) y ácido salicílico, en los que fue 0,98 (las ecuaciones de la recta de calibración de las drogas de abuso se detallan en la Tabla S3.7).

Los efectos matriz para los compuestos estudiados se calcularon obteniendo una supresión o aumento de su señal. Para las drogas de abuso, fármacos y productos de higiene personal, el efecto matriz osciló entre -88 a 66,3% para muestras de influentes, -81,2 a 67,8% para efluentes y de -86,6 a 67,1% para aguas superficiales.

Para todos los analitos, incluidos los del análisis dirigido (Tabla S3.2 y 4.3), el error de la masa exacta fue, en valores absolutos, inferior a 5 ppm, con excepción del EDDPd3 en el que fue superior. La RSD de estos errores fue inferior al 0,9%. Para el compuesto original, el porcentaje de la diferencia entra la relación del isótopo teórica y experimental fue, en todos los casos, inferior al 5%. En la identificación frente a la librería compuesta por los analitos seleccionados, se obtuvieron valores de pureza superiores a 82,4%.

Los resultados de la cuantificación realizada con el QqTOF mostraron una buena concordancia con los obtenidos mediante QqQ, en las tres matrices estudiadas como muestran las Tablas 3.2 y 4.2. Como era de esperar, las concentraciones más elevadas de las drogas de abuso correspondieron a los analitos detectados en los influentes, seguidos por los encontrados en efluentes y en aguas residuales.

Además del análisis dirigido, se realizó un análisis "no dirigido" para la identificación de metabolitos y productos de degradación de las drogas de abuso seleccionadas en ionización positiva. En este estudio se identificaron más de 25 metabolitos de las drogas de abuso tradicionalmente consumidas. Por ejemplo, en la Figura 3.3 se muestra la identificación de un metabolito de la efedrina (EPH), formado por desmetilación y oxidación, que no nos consta que se hubiera encontrado previamente en aguas residuales [fórmula propuesta $C_9H_{13}NO_2$]; en la Figura S3.3 se muestra la identificación de dos metabolitos de la cocaína (COC) descritos por MS y MS/MS formados por A) la pérdida de C_7H_4O y B) la pérdida de CH_2 y demetilación.

En el análisis "no dirigido" se abordó la identificación de supuestos compuestos utilizando una sección del software del instrumento capaz de extraer los cromatogramas correspondientes a los iones protonados y desprotonados de una lista de compuestos recogidos en una base de datos. En esta base de datos se incluyen tanto compuestos que

Las Tablas y Figuras S3. se pueden encontrar en el Supporting Information – A en http://dx.doi.org/10.1016/j.chroma.2016.07.062

responden en modo de ionización positiva (Tabla S3.4, 1212 fármacos, 546 pesticidas, 75 micotoxinas y otros compuestos), como en negativa (1212 fármacos, 546 pesticidas, 378 polifenoles y 233 micotoxinas). En modo ionización positiva se encontraron 165 fármacos, 7 pesticidas y otros 25 compuestos en influentes, 45 fármacos, 7 pesticidas y otros 3 compuestos en efluentes y 30 fármacos, 6 pesticidas y otros 5 compuestos en aguas superficiales. La Tabla S3.6 muestra el nombre, la fórmula empírica, la masa (Da), el error de masa (ppm), el ratio isotópico y la pureza de los compuestos identificados en una muestra de influente. La Figura S3.2 muestra el espectro MS y MS/MS de algunos de los compuestos identificados en una muestra de influente de depuradora. En modo ionización negativa se encontraron 86 fármacos, 2 pesticidas y otros 14 compuestos en influentes, 45 fármacos, 1 pesticidas y otros 7 compuestos en efluentes y 20 fármacos, 1 pesticida y otros 5 compuestos en aguas superficiales. La Figura 4.2 y S4.1 muestran el espectro MS y MS/MS de los supuestos compuestos hidroclorotiazida y teofilina, respectivamente.

2. Aplicación a diferentes casos de estudio

2.1 Aguas residuales

Durante 2011, 2012 y 2013, se estudió la presencia de sustancias psicoactivas en aguas residuales (Tabla 5.3), tanto en influentes como en efluentes de tres depuradoras que gestionan la mayor parte de las aguas residuales procedentes del área metropolitana de Valencia (Pinedo I, Pinedo II y Quart-Benáger) durante un periodo entre 7 – 15 días consecutivos. En este estudio se llevó a cabo el análisis de 8 drogas de abuso consideradas de consumo tradicional por la Convención de Naciones Unidas sobre Sustancias Psicotrópicas de 1971 (UNODC, 1971). El compuesto que presentó una mayor concentración en el influente de las aguas residuales fue la benzoilecgonina (BECG), el principal metabolito de excreción de la COC, a concentraciones entre un rango de 870,9 y 7752,5 ng L^{-1}. La COC inalterada fue el segundo compuesto con mayor concentración, con valores que variaron entre 110,3 – 3429,7 ng L^{-1}, seguidos por el 11-nor-9-carboxi-Δ9-tetrahidrocannabinol (THC-COOH), metabolito principal del cannabis, (18,8 – 940,2 ng L^{-1}), anfetamina (AMP) (1,7 – 110,0 ng L^{-1}), éxtasis (MDMA) (1,0 – 159,1 ng L^{-1}), ketamina (KET) (1,1 – 131,8 ng L^{-1}) y metanfetamina (MAMP) (1,2 – 69,1 ng L^{-1}). Todos ellos se detectaron en el 100% de las muestras analizadas (Tabla S5.5). La 6 acetilmorfina (6-MAM), metabolito exclusivo de la HER, sólo se encontró en una muestra (16,6 ng L^{-1}). Respecto a las concentraciones de estos compuestos en los efluentes en las campañas de 2012 y 2013 (Tabla S5.6), la BECG presentó la mayor concentración en todas las muestras

Las Tablas y Figuras S3. se pueden encontrar en el Supporting Information – A en http://dx.doi.org/10.1016/j.chroma.2016.07.062

analizadas (3,1 – 755,0 ng L^{-1}), seguida por el MDMA (1,2 – 84,7 ng L^{-1}) y la KET (1 – 43,4 ng L^{-1}). Las concentraciones detectadas en el presente estudio estuvieron en concordancia con las encontradas en otras ciudades europeas (Huerta-Fontela *et al.*, 2008; Ort *et al.*, 2014; Postigo *et al.*, 2010; van Nuijs, Mougel*, et al.*, 2011; Zuccato *et al.*, 2008)

Además, en 2013, se realizó un estudio de la presencia de 42 sustancias psicoactivas usando dos analizadores diferentes (QqTOF / QqQ), y otro estudio de 22 sustancias entre las que se analizaron fármacos, productos de higiene personal y cannabinoides. En la Tabla 3.2 se muestra la comparación de las concentraciones de las sustancias psicoactivas en influente y efluente determinadas con ambos analizadores. En el influente se hallaron un total de 7 compuestos del grupo de las anfetaminas, siendo las detectadas a mayor concentración MDMA (64 / 67 ng L^{-1}), AMP (62 / 72 ng L^{-1}) y 3,4-metilendioxianfetamina (MDA) (17 / 85 ng L^{-1}). La KET perteneciente al grupo de las arilciclohexilaminas presentó una concentración de 11 / 13 ng L^{-1}. La COC y tres metabolitos secundarios de ésta se detectaron en la muestra de influente, siendo las halladas s a mayor concentración BECG (1450 / 1593 ng L^{-1}) y COC (550 / 618 ng L^{-1}). Se detectaron 4 opiáceos, codeína (CODE) (1527 / 969 ng L^{-1}), morfina (MOR) (195 / 196 ng L^{-1}), EDDP (108 / 65 ng L^{-1}) y metadona (MET) (24 / 26 ng L^{-1}). Los cannabinoides, Δ9-Tetrahidrocannabinol (THC) y THC-COOH, se encontraron a concentraciones entre 127 / 97 ng L^{-1} y 148 / 48 ng L^{-1}, respectivamente. En el efluente de las WWTPs se hallaron 10 de las 18 drogas detectadas en el influente incluyendo 4 opioáceos, dos cocaínicos (BECG y ECME), 3 anfetamínicos (EPH, MDMA y 3,4-metilenedioxietilanfetamina (MDEA)), y además la KET. La mayoría de ellos presentaron concentraciones menores que en los influentes, a excepción de la EPH, MDEA, KET y MET. Este fenómeno puede llegar a ocurrir por varios motivos. Justificaciones plausibles de estos valores son los tiempos de residencia inferiores a 24 h, la deconjugación de los metabolitos y / o productos de transformación y la desorción de las partículas durante el tratamiento de aguas residuales.

La Tabla 4.2 muestra la comparación entre las concentraciones de las 22 sustancias analizadas en aguas residuales (fármacos, productos de higiene personal y cannabinoides), por ambos detectores (QqTOF/QqQ). De los fármacos, el naproxeno fue el detectado a mayor concentración (2963 / 3327 ng L^{-1}), seguido por el acetaminofén (2114 / 2497 ng L^{-1}) y el ibuprofeno (1796 / 1978 ng L^{-1}). De los productos de higiene personal el compuesto encontrado a mayor concentración fueron el bisfenol A (495 / 571 ng L^{-1}), propilparabén (494 / 519 ng L^{-1}) y etilparabén (99 / 113 ng L^{-1}). De los dos cannabinoides sólo se detectó el THC-COOH (409 / 592 ng L^{-1}). En el efluente se detectaron un total de 12 compuestos,

de los 19 detectados en el influente, siendo todos ellos encontrados a menor concentración a excepción de la warfarina y la indometacina.

En 2014 se realizó un estudio para determinar el etil sulfato (metabolito secundario de la ingesta de bebidas alcohólicas) en los influentes y efluentes de Pinedo I, Pinedo II y Quart-Benáger durante 17 días consecutivos, en el que se incluyeron los días en los que se celebró la festividad de las Fallas (15-19 de marzo). Las concentraciones detalladas de etil sulfato durante este periodo se muestran en las Tablas S6.4, S6.5 y S6.6, donde se observa un incremento de etil sulfato durante los fines de semana y durante los días de celebración de las Fallas. Las frecuencias, concentraciones medias y los rangos de concentración se muestran en la Tabla 6.1, siendo la concentración media detectada de etil sulfato entre 4,87 – 7,04 μg L^{-1}.

El estudio realizado en 2015, se basó en el análisis de 12 biomarcadores urinarios específicos humanos en influentes de aguas residuales de Pinedo I, Pinedo II y Quart-Benáger, para establecer cuáles de ellos son aptos para estimar el tamaño poblacional a los que abastece cada una de las WWTPs. Además, se analizaron 4 drogas de abuso para estimar su consumo. La Tabla 7.3 muestra las concentraciones de cada una de estas sustancias obtenidas por los dos métodos de extracción ensayados. Todos los analitos se detectaron con el método de extracción SPE, a excepción del 5-HIAA y creatinina que apenas fueron retenidos por la fase sólida. Por ello se optó por usar un método de análisis alternativo llamado *dilute and shoot*, ya explicado detalladamente en el apartado 1.1.1. Mediante *dilute and shoot* se detectaron 7 de los 12 compuestos analizados (5-HIAA, atenolol, cafeína, CODE, cotinina, creatinina, hidroxicotinina (OH-COT)). Las concentraciones obtenidas, para aquellos compuestos que pueden cuantificarse por ambos métodos, fueron del mismo orden. Las concentraciones de los biomarcadores urinarios específicos humanos, solamente detectados mediante la extracción SPE, estaban entre 3.30 to 30.13 μg L^{-1} para el acesulfamo, 0,002 – 0,030 μg L^{-1} para la carbamazepina, 17,57 – 37,49 μg L^{-1} para la hidroclorotiazida, 2,61 – 4,36 μg L^{-1} para el naproxeno y 9,68 y 27,28 μg L^{-1} para ácido salicílico. Dentro de las drogas de abuso, la bufotenina (BUF) se determinó en un rango de concentraciones entre 0,014 – 0,104 μg L^{-1}, el cocaetileno (CET) entre 0,013 – 0,063 μg L^{-1}, la MOR entre 0,068 – 0,217 μg L^{-1} y el THC-COOH entre 0,209 – 0,360 μg L^{-1}. Para los compuestos detectados usando ambos métodos, las concentraciones de atenolol mediante SPE fueron entre 14,34 – 46,67 μg L^{-1} y entre 15,21 – 90,11 μg L^{-1} para *dilute and shoot*. Para la cafeína 21,93 – 53,80 μg L^{-1} (SPE) y 32,98 – 98,73 μg L^{-1} para *dilute and shoot*., para la CODE 0,278 – 0,555 μg L^{-1} (SPE) y 0,304 – 0,905 μg L^{-1} (*dilute and shoot*). Para la

cotinina 1,10 – 1,89 μg L^{-1} (SPE) y 1,52 – 4,13 μg L^{-1} (*dilute and shoot*). Para creatinina, 0,200 – 0,611 μg L^{-1} (SPE) y 0,49 – 2,72 μg L^{-1} (*dilute and shoot*) y para OH–COT, 1,09 – 3,60 μg L^{-1} (SPE) y 1,89 – 3,43 μg L^{-1} (SPE). El 5-HIAA se detectó únicamente por el método *dilute and shoot* en un rango entre 5.53 – 14.31 μg L^{-1}. Las concentraciones obtenidas para estos compuestos fueron similares a los reportados por varios estudios en otros países (Boleda *et al.*, 2009; Chiaia *et al.*, 2008; Lai *et al.*, 2015; Nodler *et al.*, 2013; Senta *et al.*, 2015; Yan *et al.*, 2014)

2.2 Lodos deshidratados

Algunos de los compuestos psicoactivos fueron detectados en los lodos deshidratados procedentes de Pinedo I, Pinedo II y Quart-Benáger. La Figura 2.4 muestra los resultados obtenidos para las drogas de abuso analizadas, entre las que se encuentran la COC y sus metabolitos, opiáceos, arilciclohexilaminas, anfetamínicos y triptaminas. La COC mostró concentraciones mayores que sus metabolitos BECG y ECME. Los valores de estos compuestos detectados en Pinedo I y Pinedo II fueron ligeramente superiores a los de Quart-Benáger. Los datos no mostraron tendencia temporal, probablemente debido a que los compuestos retenidos en los lodos deshidratados de las depuradoras pueden corresponder a mezclas de varios días. En general, las concentraciones de los opiáceos fueron superiores a las de los compuestos cocaínicos. MOR, MET y CODE se detectaron en los lodos de las tres depuradoras. Otras sustancias psicoactivas presentes a bajas concentraciones fueron 4-metoxianfetamina (PMA), KET y BUF. El uso agrícola como fertilizantes de estos lodos deshidratados podría ser una posible vía de contaminación del sustrato, pudiendo producirse su filtración a las aguas subterráneas. Además, podría afectar a la germinación y crecimiento de las plantas cultivadas.

2.3 Sólidos en Suspensión

Los resultados de la cuantificación de las drogas estudiadas en los sólidos en suspensión de los influentes de las tres WWTPs, Pinedo I, Pinedo II y Quart-Benáger, se muestran en la Figura 2.3. Los cocaínicos y opiáceos fueron los compuestos más frecuentes y detectados a mayor concentración. Respecto a la COC y sus metabolitos, Pinedo I presenta una alta concentración de COC y su metabolito ECME, mientras que el metabolito cocaetilieno (COCET) sólo estuvo presente en una muestra y la BECG estuvo ausente. Por el contrario, en Pinedo II y Quart-Benáger, el metabolito más frecuente fue la BECG llegando a concentraciones de 223,1 ng g^{-1} y 119,9 ng g^{-1}, respectivamente. La COC se observó de forma esporádica en Pinedo II y la COCET en Quart-Benáger. Respecto a

los derivados morfínicos, MET y CODE fueron detectados tanto en Pinedo I como en Pinedo II, pero no en Quart-Benáger. La MET fue detectada en la mayoría de muestras, pero no presentó un patrón de concentraciones claro a lo largo de la semana. La constante presencia de MET puede deberse a su continuado uso controlado para tratar la adicción a la HER. En el caso de la CODE, su presencia fue esporádica en algunas muestras. Respecto a otras sustancias psicoactivas detectadas en los sólidos en suspensión cabe destacar la presencia de BUF (22 – 63 ng g^{-1}) y KET (46 ng g^{-1}), como ocurrió en el caso de los lodos deshidratados.

2.4 Aguas superficiales

Durante 2012 y 2013 se diseñó un muestreo a gran escala de las aguas superficiales a lo largo del río Turia, donde se analizaron 42 sustancias psicoactivas. La Tabla 8.1 resume los niveles de concentración (mínimo, máximo y media) y la frecuencia de detección de las drogas estudiadas (la Tabla S8.5 detalla las concentraciones de las drogas presentes en cada uno de los puntos de muestreo). En 2012, se detectaron 6 compuestos en un total de 22 puntos de muestreo. MDMA y 4-MeO-PCP se detectaron sólo en 1 muestra a niveles de 22,77 y 37,61 ng L^{-1}, respectivamente. BUF y MET, se detectaron en 3 puntos de muestreo en una concentración media de 29,33 ng L^{-1} y 15,20 ng L^{-1}, respectivamente. PMA se detectó en 4 puntos de muestreo en un rango entre 5,11 y 19,25 ng L^{-1} y una concentración media de 12,34 ng L^{-1}. El compuesto más frecuente fue la BECG, en una concentración media de 25,45 ng L^{-1}. En 2013, se hallaron 7 compuestos en un total de 31 puntos de muestreo, 4 de ellos presentes también en 2012. 4-MeO-PCP fue detectada en 1 punto de muestreo distinto al del 2012, a una concentración de 7,55 ng L^{-1}. MDMA apareció en 5 puntos de muestreo (media de 4,67 ng L^{-1}) y BECG y MET en 8 y 7 puntos de muestreo, respectivamente, BECG a una concentración media de 14,02 ng L^{-1} y MET a 11,42 ng L^{-1}. Además, EPH, ECME y CODE sólo se detectaron en 2013 (CODE no se analizó en 2012). ECME sólo se encontró en 1 punto de muestreo a una concentración de 15,03 ng L^{-1}. EPH y CODE se detectaron en 3 puntos de muestreo siendo los niveles de concentración de 11,60 (5,28 – 17,65) ng L^{-1} para EPH y 91,31 (81,52 – 101,02) ng L^{-1} para CODE.

2.5 Sedimentos depositados por aguas superficiales

Se analizaron cinco muestras de sedimentos depositados por las aguas superficiales del río Turia (P3, P9, P20, P26 y P27, ver localización en Figura S8.1), donde se observó la presencia de COC, BECG, MET y 4-MeO-PCP (Figura 2.5). COC y 4-MeO-PCP se

detectaron en una muestra en concentraciones de 30 ng g^{-1} y 1,33 ng g^{-1}, respectivamente. BECG se detectó en dos muestras en concentraciones de 0,94 y 0,96 ng g^{-1}. La MET estuvo presente en todas las muestras en un rango de concentraciones entre 0,29 ng g^{-1} y 0,53 ng g^{-1}.

2.6 Matrices alimentarias

Las muestras de leche no presentaron contenido en THC ni ninguno de sus metabolitos (11-hidroxi- Δ9- tetrahidrocannabinol (THC-OH), THC-COOH). Sólo el THC se detectó en 3 de las 5 fórmulas de crecimiento y en las semillas de cáñamo a concentraciones que variaron entre 4,76 ng g^{-1} y 56,11 ng g^{-1} para las fórmulas de crecimiento. La Figura 10.3A muestra un cromatograma obtenido para una muestra positiva en THC en leche de crecimiento. La ingesta recomendada de leche para niños (fórmula de crecimiento o incluso leche) es de dos raciones (400 – 500 mL por día) (Galiano *et al.*, 2013). Por lo tanto, la ingesta diaria de THC a través de este tipo de leche podría alcanzar hasta 2,38 – 28,05 ng. En la Figura S10.4 se muestra la concentración obtenida de THC en semillas de cáñamo, 0,82 mg THC kg^{-1}. En todas las muestras analizadas se detectaron concentraciones inferiores al límite establecido por la EFSA de 12 mg THC kg^{-1} (EFSA, 2011).

Respecto a las muestras de hígado no se observó la presencia ni del THC ni de sus metabolitos. La Figura 10.3B muestra el cromatograma obtenido para el extracto de hígado, donde aparecen algunos picos con la misma transición que el THC y en el mimo tiempo de retención, pudiéndose deber a la presencia de otros metabolitos no considerados en el estudio, ya que el THC puede conjugarse dando lugar a diferentes metabolitos a través de su unión con ácidos de cadena larga como el oleico o el estérico (Grotenhermen, 2003; Huestis, 2007).

3. Estimación del consumo de drogas de abuso y alcohol: *Epidemiología de alcantarilla*

La Figura 5.2 muestra la tasa media de consumo calculada en las tres WWTPs seleccionadas en 2011, 2012 y 2013 de cada una de las drogas de abuso, expresadas como mg $día^{-1}$ 1000 $habitantes^{-1}$. Pinedo I solo recibe las aguas residuales de la población de Valencia, la tercera ciudad con mayor población de España, con numerosos lugares de ocio y una intensa vida nocturna. Pinedo II y Quart-Benáger, reciben también parte de las aguas residuales de la población de Valencia y su área metropolitana. Tras realizar el estudio se observó que las drogas más consumidas eran la COC y el cannabis. El consumo estimado

de COC en 2011 fue 1641,3, 1181,7 y 1332,6 mg día^{-1} 1000 habitantes^{-1} en Pinedo I, Pinedo II y Quart-Benáger, respectivamente. En 2012, el consumo de COC decreció y esta tendencia de descenso se confirmó en 2013 en las tres depuradoras. El mayor consumo de COC en las campañas de 2011 y 2013 fue en Pinedo I, lo que podría estar relacionado con los hábitos urbanos de Valencia. El consumo estimado de COC en las tres WWTPs fue similar al observado en 42 municipios de Cataluña (España) y Londres (Reino Unido) (Huerta-Fontela *et al.*, 2008; Zuccato *et al.*, 2008) y superior a otros territorios como Milán (Italia) Lugano (Suiza), Bruselas (Bélgica) y Gales del Sur (Reino Unido) (Kasprzyk-Hordern *et al.*, 2009; van Nuijs, Mougel*, et al.*, 2011; Zuccato *et al.*, 2008). Los valores medios de consumo en los tres años en cuanto a dosis fueron 13,7, 11,1 y 13,3 dosis día^{-1} 1000 habitantes^{-1} de COC en Pinedo I, Pinedo II y Quart-Benáger, respectivamente (una dosis estándar equivale a 100 mg) (Terzic *et al.*, 2010; Zuccato *et al.*, 2008).

El consumo estimado de cannabis fue siempre más alto en Pinedo I que en las otras WWTPs (4034,4, 4163,2 y 12422,5 mg día^{-1} 1000 inhabitantes^{-1} en 2011, 2012 and 2013, respectivamente), sufriendo un gran ascenso en 2013 en las tres depuradoras. El consumo estimado en 2011 y 2012 en Pinedo II (1935,0 y 1579,6 mg día^{-1} 1000 habitantes^{-1}, respectivamente) y Quart-Benáger (1880,0 y 1743,4 mg día^{-1} 1000 habitantes^{-1}, respectivamente) fue menor que en Zagreb (Croacia), 15 municipios de Cataluña (España), Milán (Italia), Lugano (Suiza) y Londres (Reino Unido) (Boleda *et al.*, 2009; Terzic *et al.*, 2010; Zuccato *et al.*, 2008). El consumo estimado en 2013 en Pinedo I, Pinedo II y Quart-Benáger fue mucho mayor que en las ciudades mencionadas anteriormente (12422,5, 10591,8 y 9419,8 mg día $^{-1}$ 1000 habitantes^{-1} respectivamente). Las dosis equivalentes para Pinedo I, Pinedo II y Quart-Benáger fueron de 55,0, 37,6 y 34,8 dosis día^{-1} 1000 habitantes^{-1}, respectivamente (dosis de 125 mg). Las tendencias temporales desde 2011 a 2013 indicaron un descenso en el consumo de COC a la vez que un incremento del cannabis. La misma tendencia observó en un estudio realizado en numerosas ciudades europeas durante 2014 (Ort *et al.*, 2014).

El uso recreacional de la KET es difícil de determinar debido a su utilización como anestésico en los hospitales y a su uso veterinario. El consumo estimado más alto en 2012 se detectó en Pinedo I (240,4 mg día^{-1} por 1000 habitantes^{-1}) y en 2013 en Pinedo II (694,8 mg al día^{-1} por 1000 habitantes^{-1}). Los valores detectados en las tres WWTPs se incrementaron en 2013 siendo el aumento registrado en Pinedo II y Quart-Benáger mayor que en Pinedo I. Esto podría significar un aumento en el consumo de KET como droga de

abuso frente a su uso farmacéutico, ya que en este último se considera que es bastante estable a lo largo del tiempo.

Pinedo I fue la WWTP donde se estimó el mayor consumo de AMP en las tres campañas (135,5, 97,5 y 70,8 mg día^{-1} 1000 habitantes^{-1} en 2011, 2012 y 2013, respectivamente). Estos valores fueron similares a los reportados en 42 municipios Cataluña (España), Londres (Reino Unido), Bruselas (Bélgica) (Huerta-Fontela *et al.*, 2008; van Nuijs, Mougel*, et al.*, 2011; Zuccato *et al.*, 2008). La tendencia de consumo de AMP durante los tres años fue decreciendo hasta 2013, cuando el valor más bajo se detectó en Pinedo II (29,9 mg al día^{-1} 1000 habitantes^{-1}), aunque el valor más bajo detectado en las tres campañas fue en Quart-Benáger en 2012 (2,2 mg día^{-1} por 1000 habitantes^{-1}). Las dosis de AMP habitualmente consumidas son de 50 mg (Terzic *et al.*, 2010), por lo que las dosis promedio correspondientes a Pinedo I, Pinedo II y Quart-Benáger fueron 2,03, 1,21 y <1 dosis día^{-1} 1000 habitantes^{-1}.

Asimismo, el consumo de MAMP disminuyó de 2011 a 2013, de 45,5 a 3,9 en Pinedo I, de 30,3 a 2,8 en Pinedo II y de 17,5 a 1,3 mg día^{-1} 1000 habitantes^{-1} en Quart-Benáger. Como en el caso de la AMP, se detectaron los valores más altos en Pinedo I y el más bajo en Quart-Benáger. Los valores de 2013 fueron próximos a los valores detectados en varios municipios de Cataluña (España) (Postigo *et al.*, 2010). El consumo en de esta droga en Valencia en 2011 y 2012 (33,8 y 30,4 mg al día^{-1} por 1000 habitantes^{-1} en Pinedo I y Pinedo II, respectivamente) fue mayor que los reportados para Milán (Italia) o Londres (Reino Unido) (Zuccato *et al.*, 2008). La media en el consumo estimado de MAMP (cada dosis de aproximadamente de 40 mg) fue <1 dosis al día^{-1} 1000 habitantes^{-1} respectivamente en las tres WWTPs (Postigo *et al.*, 2010).

Con respecto al MDMA, el consumo estimado más alto fue en Pinedo I (9,2, 9,0 y 21,9 mg al día^{-1} 1000 habitantes^{-1} en 2011, 2012 y 2013, respectivamente). Los valores más bajos fueron en Quart-Benáger (2,9, 0,2 y 10,1 mg al día^{-1} 1000 habitantes^{-1} en 2011, 2012 y 2013 respectivamente). Estos valores están en concordancia con los detectados en Lugano (Suiza), Milán (Italia), Londres (Reino Unido) y Zagreb (Croacia) (Terzic *et al.*, 2010). En contraste con lo que sucede con COC, AMP y MAMP, el consumo de MDMA aumentó en 2013 en las tres WWTPs. El promedio correspondiente en términos de dosis fue <1 dosis día^{-1} 1000 habitantes^{-1} en las tres WWTPs, considerando la dosis de MDMA de 100 mg (Postigo *et al.*, 2010).

Se realizó un análisis estadístico ANOVA para determinar si las diferencias en el consumo entre las tres WWTPs eran estadísticamente significativas. De acuerdo con los datos obtenidos en particular para el cannabis y la COC, Pinedo I muestra un elevado consumo que podría ser debido a que esta WWTP sólo trata el agua de la ciudad Valencia, con mucha vida social y nocturna. De hecho, mucho más que las otras ciudades circundantes, que son sólo ciudades dormitorio o áreas industriales. Sin embargo, las diferencias observadas entre las tres WWTPs no fueron estadísticamente significativas.

El consumo diario de las drogas de abuso en las 3 campañas en el influente de Pinedo I, Pinedo II y Quart-Benáger se ilustra en la Figura 5.3. La COC y el MDMA muestran una clara tendencia recreativa, ya que sufren un aumento de consumo durante el fin de semana. Se realizó un estudio estadístico para establecer diferencias de consumo entre los días de la semana (de lunes a jueves) y los días correspondientes al fin de semana (de viernes a domingo). Estas diferencias fueron estadísticamente significativas para COC, MDMA y AMP ($p < 0,05$), pero no para KET, MAMP y cannabis. Estas tendencias son consistentes con las reportadas en otros países (Huerta-Fontela *et al.*, 2008; Terzic *et al.*, 2010)

Los coeficientes de COC/BECG calculados, considerando el patrón de metabolismo y excreción de COC, oscilaban entre 0,3 y 0,7 (Postigo *et al.*, 2010; Van Nuijs *et al.*, 2009), dependiendo de condiciones ambientales tales como temperatura o tiempo de residencia. Los datos obtenidos en el presente estudio se resumen en la información complementaria Figura S5.1. En 2011 y 2012, la relación media obtenida está dentro del rango (entre 0,2 y 0,5). Sin embargo, 2 muestras de influentes recogidas en 2013 en Quart-Benáger y Pinedo II mostraron inesperadamente altos cocientes COC/BECG (2,0 el viernes y 1,6 el domingo, respectivamente). Además, el sábado, las tres WWTPs presentan una proporción cercana a 1. Todos estos hallazgos indicaron una posible eliminación de COC en el sistema de alcantarillado sin haber sido consumida (BECG constante, aumento en COC). El jueves, de acuerdo con los periódicos (HortaNoticias.com, 2013) la policía española desmanteló un punto de venta de COC situado en una casa de Manises (cubierta por Quart-Benáger). Eso también podría tener una influencia en otras partes de la ciudad debida a varias intervenciones policiales. El consumo no regular de COC también fue observado por Bijlsma et al. (2012). Otros enfoques, como el análisis quiral, pueden ayudar en estas identificaciones demostradas por Baker et al. (2012).

Los datos aquí presentados sobre el consumo de drogas de abuso en Valencia en los años del 2011 al 2013, así como los datos de las campañas del 2014 al 2016 se enmarcan dentro de una acción europea en la que participan más de 40 ciudades pertenecientes a más de 20 países diferentes. Cada año, el EMCDDA (Observatorio Europeo de las Drogas y las Toxicomanías) revela los resultados de los distintos patrones geográficos y temporales del consumo de las drogas de abuso a través de publicaciones y mapas interactivos en su página web (EMCDDA, 2016).

La estimación del consumo de alcohol se determinó durante 17 días consecutivos (4 – 20 de marzo 2014), abarcando los días de la festividad de las Fallas (15-19 de marzo de 2014). Los consumos detallados de alcohol se resumen en la Tabla 6.2. Los datos obtenidos en este estudio oscilaron entre 1,11 a 23,81 mL día^{-1} habitantes^{-1} en Pinedo I, de 1,07 a 9,07 mL día^{-1} habitantes^{-1} en Pinedo II y de 3,31 a 56,11 mL día^{-1} habitantes^{-1} en Quart-Benàger. Estos resultados están de acuerdo con los datos obtenidos en Santiago de Compostela, Barcelona y Oslo (Mastroianni *et al.*, 2014; Reid *et al.*, 2011; Rodríguez-Álvarez *et al.*, 2014). Una vez más, se observa un mayor incremento en el consumo de alcohol durante los fines de semana que durante los días laborables, durante una semana considerada normal. Los resultados obtenidos muestran un comportamiento excepcional y una evidencia clara del incremento en el consumo de alcohol desde el comienzo de la festividad de las Fallas. Las diferencias entre el fin de semana (20,88 mL día^{-1} habitantes^{-1}) y los días laborables (19,98 mL día^{-1} habitantes^{-1}) no se observaron durante la festividad. Los valores máximos se alcanzaron el último día de celebración de las Fallas, el miércoles 19 de marzo, denominado "Nit de la Cremà", valores de 23,81, 9,07 y 56,11 mL día^{-1} habitantes^{-1} en Pinedo I, Pinedo II y Quart-Benáger respectivamente. La Figura S6.2 muestra el consumo diario de alcohol. Según los datos de 2010 (WHO, 2014), el consumo de alcohol se distribuye en 50% de cerveza, 28% de bebidas con alta graduación, 20% de vino y 2% de otros. Teniendo en cuenta que la cerveza, las bebidas con alta graduación y el vino tienen 5, 40 y 12% de alcohol puro, respectivamente, y que los volúmenes más comunes de estas bebidas son 250 ml de cerveza, 30 ml de alcohol y 125 ml de vino, cada uno contiene unos niveles de 12,5, 12 y 15 ml de etanol, respectivamente. De esta manera podemos estimar el número de las bebidas que consumen la población. Durante una semana normal, el consumo de alcohol, en días laborables es aproximadamente menos de la mitad de una porción de cada bebida, 85, 10,5 y 35 mL día^{-1} habitantes^{-1} de cerveza, alcohol y vino, respectivamente; mientras que durante el fin de semana es un poco más de la mitad de la ración de cerveza, alcohol o vino (147, 18,4 y 61,3 mL día^{-1} habitantes^{-1}, respectivamente). El consumo de alcohol aumenta

marcadamente en la semana de las Fallas, alcanzando casi dos porciones de cerveza (400 mL día^{-1} habitantes^{-1}) o una porción y media de alcohol o vino (50 y 166,7 mL día^{-1} habitantes^{-1}, respectivamente). Según el Informe de situación mundial sobre el alcohol y la salud 2014 de la Organización Mundial de la Salud, el consumo medio estimado de alcohol por habitante (población > 15 años) en España es de 16,4 L de etanol puro al año, aproximadamente 45 mL día^{-1} habitantes^{-1}. Se puede originar una pequeña desviación porque este estudio se limita a Valencia, mientras que el informe de la Organización Mundial de la Salud es para todo el estado español (WHO, 2014).

En 2015 se realizó un estudio de la estimación del consumo de 4 drogas de abuso, cannabis, COC, BUF y HER. El consumo se determinó usando dos métodos para el establecer el tamaño poblacional, uno mediante la utilización de biomarcadores urinarios específicos humanos (cafeína, cotinina, 5-HIAA) y otro mediante la utilización de los parámetros tradicionales (COD, BOD, capacidad de la WWTP, N total y P total). Los promedios de consumo de estas cuatro drogas de abuso se resumen en la Tabla 7.4.

En Quart-Benáger, las tendencias de consumo de las cuatro drogas de abuso son similares, con todas las estimaciones de la población realizadas, excepto en la que se utilizó la cafeína. En Pinedo I, los datos de consumo obtenidos a partir de las estimaciones de la capacidad de la WWTP, cafeína y cotinina, están en concordancia, mientras que los realizados con las cargas de 5-HIAA y COD muestran una tendencia diferente, con un mayor consumo durante los fines de semana. En Pinedo II, los resultados de consumo fueron similares, excepto con aquellos datos estimados a partir de la COD, que fueron ligeramente diferente del resto. En Pinedo I y II, hubo una fuerte correlación entre los promedios de las estimaciones de consumo realizadas a partir de las cargas de cafeína y cotinina, y la capacidad de la WWTP, con una diferencia de sólo 0,3 y 1 g 1000 habitantes^{-1} para Pinedo I y II, respectivamente. La cantidad promedio de las drogas de abuso consumidas en Pinedo I y II es similar, pero superior a la de Quart-Benàger.

Estas estimaciones del consumo de drogas presentaron ciertas limitaciones debido a los metabolitos que se utilizaron para este fin. Muchos de ellos han sido señalados por el Grupo de Análisis de Aguas Residuales de Europa (SCORE) (NIVA, 2016), otros también se destacan en una serie de revisiones (Evgenidou *et al.*, 2015; van Nuijs, Castiglioni*, et al.*, 2011). Las cargas de THC-COOH en las aguas residuales, basadas en los datos de excreción de orina, pueden presentar cierto grado de error, ya que la mayor parte del THC se excreta a través de heces, lo que puede transformar a THC-COOH conduciendo a una

sobreestimación del consumo de THC. La MOR también puede aparecer en las aguas residuales debido al metabolismo de otras drogas/fármacos que se metabolizan a MOR y como la propia MOR, por tanto la MOR para determinar el consumo de HER puede sobrestimar el consumo real de esta droga (Evgenidou *et al.*, 2015). Por otra parte, la COC se estima comúnmente a partir de BECG, metabolito más estable de la COC, pero en este estudio se realizó a partir del metabolito COCET, que es un metabolito de la COC que se produce cuando se consume conjuntamente con alcohol. Sin embargo, la mezcla recreativa de alcohol y drogas ha aumentado dramáticamente en las últimas décadas, por lo que lo hemos considerado una estimación del consumo de COC apropiada. La estimación de las cargas consumidas de las drogas ilícitas en este estudio se ha comparado con las estimaciones de consumo de drogas de abuso realizadas en Valencia y su área metropolitana durante 2011, 2012 y 2013, descritas anteriormente. El consumo de cannabis fue siempre superior en Pinedo I (4034,4, 4163,2 y 12422,5 m mg día^{-1} 1000 habitantes^{-1} en 2011, 2012 y 2013, respectivamente) que en Pinedo II (1935,0, 1579,6 y 10591,0 mg día^{-1} 1000 habitantes^{-1}) y Quart-Benáger (1880,0, 1743,4 y 9419,8 mg día^{-1} 1000 habitantes^{-1}). Este consumo sufrió un gran aumento en 2013 en las tres WWTPs. Los datos de 2015 muestran una tendencia estable en el consumo ya que los resultados son muy similares a los de 2013. La tendencia observada en el consumo de COC en los años 2011 – 2013, se ratificó en 2015, aunque en este caso la estimación de la COC se llevó a cabo a partir de un metabolito diferente. El consumo estimado de COC en 2011 fue de 1641,3, 1181,7, 1332,6 mg día^{-1} 1000 habitantes^{-1} en Pinedo I, Pinedo II y Quart-Benàger, respectivamente, que disminuyeron a 1191,3, 972,8 y 1034,9 mg día^{-1} 1000 habitantes^{-1} en 2015 (Figuras S7.11-S7.13), aunque calculado usando una metabolito diferente, los datos reflejan una tendencia decreciente. El consumo de HER a través de la MOR no se estimó debido al problema con la sobreestimación, y la BUF, como nueva sustancia psicoactiva, no fue cubierta en nuestro estudio anterior.

4. Biodegradación estereoselectiva en microcosmos

4.1 Ensayos de microcosmos en aguas superficiales

La biotransformación de la FL en las aguas superficiales se estudió a una concentración de 1 μg L^{-1} de la mezcla racémica y revela que la FL sufre degradación fotoquímica y microbiológica (Figura 9.3 y Tabla S9.7). La fotólisis se considera el fenómeno más importante, ya que el 74,5% de la (*S*) -FL y el 79,2% (*R*) -FL se eliminaron en condiciones abióticas y luz (LAR). Se observó que este proceso, como se esperaba, no

era estereoselectivo. Los procesos microbianos mostraron una ligera enantioselectividad hacia el enantiómero (*R*) y condujeron a la eliminación del 60,4% de la (*S*) -FL y 67,9% (*R*) -FL en condiciones bióticas y oscuridad (DBR). Como se esperaba, el reactor en condiciones bióticas y luz (LBR) utilizando procesos fotoquímicos y microbianos condujeron a la mayor eliminación de FL: 98,4% de (*S*) -FL y 96,7% de (*R*) -FL. Las condiciones abióticas y oscuridad (DAR) no condujeron a ninguna degradación de FL, lo que indica que no hay contribución significativa de la sorción de FL, por ejemplo en las partículas en suspensión. Se observaron trazas de NFL tanto en condiciones abióticas como bióticas (Figura S9.5). Esto indica que la degradación de FL que conduce a la formación de NFL, se produce durante los procesos tanto fotoquímicos como microbianos.

4.2 Ensayos de microcosmos en lodos activos

Los estudios de degradación en microcosmos de lodos activos se llevaron a cabo en la oscuridad, produciéndose la transformación estereoselectiva de la FL en NFL debida a la prevalencia de los procesos metabólicos microbianos en estos biorreactores. La transformación de la FL se estudió a dos concentraciones: 10 y 100 μg L^{-1} de la mezcla racémica de FL (Figura 9.4 y Tabla S9.8). En ambos casos se observó una disminución significativa en la concentración de (*S*) -FL y (*R*) -FL. En el microcosmos fortificado con 10 μg L^{-1}, la eliminación de la FL se produjo rápidamente durante los primeros 30 minutos (50% de degradación). Este proceso no fue estereoselectivo (EF 0,5) y no condujo a la esperada formación de NFL, lo que permite hipotetizar que esta rápida eliminación de FL se debe a su sorción en los sólidos en suspensión. A continuación, la degradación de FL se enlenteció y condujo a su transformación estereoselectiva favoreciendo la degradación (*S*) -FL (EF < 0,3) y a la formación de NFL enriquecida con su (*S*)-enantiómero (EF 0,7). El rendimiento en porcentaje molar de la formación de NFL fue del 10,7% para (*S*) -FL y del 6,2% para (*R*) -FL.

Los ensayos de microcosmos realizados con 100 μg L^{-1} de FL proporcionaron resultados similares pero el efecto de sorción durante los 30 min iniciales no se observó. Esto se debe probablemente a una carga inicial de FL mucho mayor en el biorreactor de 100 μg L^{-1} que no permitiría que se registre dicho cambio. Los procesos microbianos estereoselectivos produjeron una transformación de la FL del 60% con una preferencia dos veces mayor hacia (S) -FL (EF < 0,3), la eliminación del 80% de (S) -FL y sólo 38% de eliminación de (*R*) -FL y la formación de NFL enriquecida en el (*S*) -enantiómero (EF >

0,7). El rendimiento en porcentaje molar de la formación de NFL indicó un 11,7% para (*S*) -FL y 7,4% y (*R*) –FL, lo que corrobora los resultados obtenidos para el biorreactor adicionado con 10 μg L^{-1}. Curiosamente en ambos biorreactores, se observaron largas fases de latencia sin una apreciable degradación de la FL (3h y 2h en el caso de biorreactor 10 μg L^{-1} y 100 μg L^{-1} FL, respectivamente).

Los estudios cinéticos (Tabla 9.1) confirmaron la poca biodegradación de la FL y la naturaleza más recalcitrante de *(R*) -FL en comparación con la (*S*) en el biorreactor de 100 μg L^{-1}. Las costantes K_{biol} y $t_{1/2biol}$ de la transformación de la (*S*) -FL fueron 0,04 $LgSS^{-1}h^{-1}$ y 19h. K_{biol} y $t_{1/2biol}$ de cinética de degradación de la (*R*) -FL fueron de 0,01 $LgSS^{-1}h^{-1}$ y 68 h. Debido a la falta de degradación de (*R*) -FL en el biorreactor de 10 μg L^{-1}, no se pudieron realizar estudios cinéticos.

Los resultados anteriores indicaron que para facilitar la degradación de la FL durante el tratamiento de las aguas residuales se necesitan tiempos de retención en los lodos más largos. Sin embargo, estos procesos son estereoselectivos y podrían conducir al enriquecimiento del enantiómero más recalcitrante, así como la formación de metabolitos biológicamente activos.

Esto contrasta con los procesos metabólicos en humanos, que favorecen la degradación del enantiómero (*R*) y conducen al enriquecimiento de FL en la orina con el enantiómero (*S*) (Caccia, 1998). De hecho, en un estudio realizado en aguas residuales no tratadas a gran escala, la FL se enriqueció ligeramente con el enantiómero (*S*) (EF 0,68) (Petrie *et al.*, 2016). Otros estudios evidenciaron que la FL se enriquece con el enantiómero (*S*) en aguas residuales (EF > 0,60) y en ríos contaminados (V. K. Barclay *et al.*, 2012; Evans *et al.*, 2015; López-Serna *et al.*, 2013; Ma *et al.*, 2016). Barclay et al. 2012 no observaron una estereoselectividad significativa durante los procesos de tratamiento de aguas residuales (V. K. H. Barclay *et al.*, 2012). Por el contrario, Ribeiro et al. 2014 detectaron (*R*) -FL en los efluentes de las WWTP lo que indicaba una rápida degradación de la (*S*) -FL durante el proceso de biológico de degradación que se lleva a cabo en las WWTP. Estos resultados estuvieron de acuerdo con los obtenidos por MacLeod et al. (2007) que verificaron el enriquecimiento con (*R*) -FL. Los resultados de los estudios de metabolismo humano, así como las mediciones realizadas en las distintas etapas del

tratamiento de aguas residuales, a escala completa o considerando solo el microcosmos, indican que el porcentaje de cada enantiómero de la FL puede depender de la composición y estructura de las comunidades microbianas presentes en las aguas residuales. Esto confirma, una vez más, la complejidad de los procesos ambientales y refuerza la necesidad de realizar estudios más amplios centrados en la transformación de los contaminantes quirales en el medio ambiente.

5. Evaluación del riesgo ecotoxicológico de las drogas de abuso

En la presente tesis se han realizado diversos estudios de evaluación del riesgo ecotoxicológico de diferentes sustancias psicoactivas, mediante estudios *in silico* e *in vivo*. Los estudios teóricos de toxicidad se realizaron en base a las concentraciones de los compuestos detectados en efluentes y en aguas superficiales.

Se evaluó el riesgo ecotoxicológico de las drogas de abuso en distintos niveles tróficos calculando el cociente de riesgo (RQ), que se ha convertido en un parámetro ampliamente utilizado para dicho menester. Los valores de toxicidad aguda se utilizaron para predecir la concentración sin efecto observable (PNEC), para cada droga de abuso detectada en los efluentes de las WWTPs y en las aguas superficiales. El RQ se calculó para para los 3 niveles tróficos del ecosistema (algas, dáfnidos y peces). Los resultados obtenidos en el caso de las drogas de abuso detectadas en los efluentes se resumen en la Tabla S5.7. Según la clasificación RQ ("baja" de 0,01 a 0,1, "medio" de 0,1 a 1 y "alto"> 1), la MDMA podría plantear un riesgo medio porque el valor más alto para RQ fue 0,4 (dáfnidos). En cuanto a la KET el valor más alto de RQ fue 0,06 (algas) por lo que podría plantear un riesgo bajo. La BECG no planteó riesgo, ya que su RQ era 0,0 en todos los escenarios. Estos datos se calcularon en el efluente de las WWTPs, por lo que se planteó el peor escenario posible debido a que los efluentes se diluyen aún más en las aguas superficiales. La Tabla 8.3 resume los valores de RQ de las drogas detectadas en las aguas superficiales para los tres niveles tróficos. Según el RQ calculado, los compuestos detectados en el río Turia no representan riesgos agudos para los organismos acuáticos, ya que los RQs fueron siempre inferiores a 1. Sin embargo, las drogas de abuso se pueden considerar como contaminantes pseudopersistentes, que pese a degradarse rápidamente, tienen la capacidad de persistir en el medio ambiente dado su continuado aporte en él, por lo que la exposición crónica a estos compuestos puede producir numerosos e indeterminados efectos a largo plazo, que no pueden preverse trabajando con datos sobre la toxicidad aguda. Estos efectos pueden inducir cambios metabólicos o reproductivos en diversos organismos, toxicidad

por sinergia o efectos añadidos en organismos acuáticos, así como otros efectos tóxicos secundarios, aún no estudiados.

Los estudios *in vivo* se llevaron a cabo para evaluar la toxicidad de la FL, la cual es conocida por ser tóxica para varias especies acuáticas en concentraciones ambientalmente relevantes (Brooks *et al.*, 2003; De Andrés *et al.*, 2009; Foran *et al.*, 2004; Gonzalez-Rey *et al.*, 2013). Tanto la FL como la NFL están incluidas en la lista de los 10 fármacos potencialmente peligrosos para el medio ambiente (Ribeiro *et al.*, 2014). A pesar de la diferente potencia tóxica de los enantiómeros de FL y NFL y sus diferentes destinos ambientales, los estudios que verifican si la toxicidad de la FL y la NFL es enantioméricamente dependientes son todavía muy escasos, con resultados contradictorios y poco concluyentes.

Stanley *et al.* (2007) evaluaron la enantioespecificidad de las respuestas subletales estandarizadas y comportamentales de *P. promelas* (pruebas de 7 días) y *D. magna* (pruebas de 21 días) de FL. Se observó que (*S*) -FL era más tóxico para *P. promelas*, porque su metabolito activo principal, (*S*) -NFL, es más potente que la (*R*) -FL. Curiosamente, no se observó enantioselectividad en las pruebas agudas de 42 h en *P. promelas*. En contraste con el estudio de *P. promelas* (durante 7 días), no se observó enantioselectividad en *D. magna*. Esta respuesta enantiospecífica diferencial entre organismos modelo puede haber resultado de una homología más estrecha entre mamíferos y peces que entre mamíferos y crustáceos (Stanley *et al.*, 2007). De Andres et al. (2009) observaron una EC_{50} para (*R*) -FL de 30,5 mg L^{-1} y de 3,2 mg L^{-1} para (*S*) -FL en *T. thermophila*. Estos valores indicaron cierta enantioselectividad en la toxicidad de dichos enantiómeros con una sensibilidad del protozoo hacia la (*S*) -FL (De Andrés *et al.*, 2009).

En este estudio, el $EC50_{48h}$ para los enantiómeros de la FL en *D. magna* fue de 3,6 mg L^{-1} (S) -FL y 4,1 mg L^{-1} para (*R*) -FL. Estos valores son del mismo orden de magnitud que los descritos por Minguez et al. (2014) y Christensen et al. (2007) y están en buen acuerdo con otros estudios como el de De Andrés et al. (2009). La $EC50_{48h}$ para la NFL en a *D. magna* fue de 2,8 mg L^{-1} para (*S*) -NFL y 2,9 mg L^{-1} para (*R*) -NFL (los datos brutos se muestran en la Tabla S9.9). Los resultados indican una diferencia obvia entre la toxicidad de FL y NFL, siendo NFL más tóxica que FL, pero no se observó enantioselectividad significativa. Además, las $EC50_{24h}$ para *T. thermophila* fueron 35,3 mg L^{-1}, 1,3 mg L^{-1} y 0,9 mg L^{-1} para (*S*) -FL, (*R*) -FL y una mezcla de los enantiómeros FL (EF 0,3), respectivamente. Estos resultados contradicen los publicados por De Andres et al.

(2009), ya que dentro de ese estudio se observó que el enantiómero (*S*) era más tóxico con una $EC50_{24h}$ de 3,2 mg L^{-1} en comparación con el enantiómero (*R*) con una $EC50_{24h}$ de 30,5 mg L^{-1}. Para mayor precisión se analizaron las soluciones madre y las celdas de ensayo para confirmar la concentración antes y después del ensayo y se investigó si se observaron cambios durante el ensayo. Los resultados mostraron que los cambios en las concentraciones de los tóxicos eran mínimos durante la prueba, sin embargo, apoyan el uso del enantiómero correcto en esta prueba.

Los resultados también sugieren un efecto sinérgico entre los dos enantiómeros de la FL en la mezcla no racémica (EF 0,3), aunque esto deberá confirmarse y explorarse con estudios adicionales. Hasta la fecha, no se conoce otro estudio que haya comparado la $EC50_{24h}$ de los enantiómeros y una mezcla de los dos. El $EC50_{24h}$ para NFL indicó 3,8 mg L^{-1}, 5,9 mg L^{-1}, 0,5 mg L^{-1} para (*S*) -NFL, (*R*) -NFL y una mezcla de los enantiómeros NFL (EF 0,3). A diferencia de FL, el enantiómero (*S*) es más tóxico para la NFL. (*R*) -NFL es cuatro veces menos potente que (*R*) -FL. El $EC50_{24h}$ de la mezcla no racémica (EF 0,3) enantiómeros NFL también sugiere un efecto sinérgico. Se llevó a cabo una prueba final que contenía una mezcla de los enantiómeros FL y NFL (FL, EF 0,3; NFL, EF 0,3). La FL utilizada en el ensayo (FL, EF 0,3) se basó en un enriquecimiento del enantiómero (*R*) tal como se observa en los procesos de tratamiento activos. Esta es una consideración importante ya que este organismo forma parte de la comunidad microbiana que participa en este proceso. La Figura 9.5 y las Tablas S9.10-S9.23 muestran los datos de $EC50_{24h}$ para *T. thermophila*.

Los resultados anteriores indican que los estudios toxicológicos tradicionales que no reconocen la importancia de la estereoquímica pueden no revelar el verdadero impacto toxicológico resultante de la estereoquímica de los fármacos quirales. Los resultados indicaron que (*S*) -FL se degrada preferentemente en microcosmos de lodos activos. Estos resultados fueron los esperados, ya que (*S*) -FL es el menos tóxico de los cuatro enantiómeros FL/NFL estudiados para los protozoos (organismos que se sabe que son contribuyentes clave al proceso de tratamiento de lodos activos). Desafortunadamente, esto también indica que la FL, debido a la degradación metabólica preferencial de *(S)* -FL, se enriquece con el más tóxico (*R*) -FL. Esta acumulación de (*R*) -FL podría tener efectos perjudiciales sobre el rendimiento de los procesos de tratamiento de lodos activos.

Se puede asumir que, si no se considera la estereoquímica de la FL, la disminución de la concentración de esta como resultado del tratamiento con lodos activos conduce a un

menor impacto biológico. No obstante, este enfoque (tal como se aplica actualmente en la Evalución del Riesgo Ambiental (ERA)) puede conducir a conclusiones falsas que pueden afectar a la salud ambiental. Nuestro estudio demostró que, a pesar de la disminución general de la concentración de FL, la acumulación del tóxico (*R*) -FL y la formación del tóxico (*S*) -NFL en los lodos activos, probablemente conducirán a mayores efectos toxicológicos, como se observa en el caso de los protozoos.

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CONCLUSIONES GENERALES

CONCLUSIONS

D'acord amb els objectius de la present Tesi Doctoral, la recerca duta a terme i els resultats descrits en els capítols previs, es pot concloure que:

Primer. La cromatografia líquida acoblada a l'espectrometria de masses en tàndem, utilitzant un triple quadrupol, és selectiva, fiable, eficaç i presenta una alta sensibilitat per a la detecció sistemàtica de les drogues d'abús seleccionades en matrius complexes. Addicionalment, els espectròmetres de masses d'alta resolució, com el sistema híbrid quadrupol temps de vol, no solament determinen les drogues seleccionades, sinó que també identifiquen un gran nombre d'altres compostos no seleccionats, metabòlits i productes de degradació presents en mostres d'aigües residuals i superficials.

Segon. L'aplicació de les metodologies analítiques desenvolupades mostra que, en els influents de les estaciones depuradores d'aigües residuals, es van detectar concentracions de ng L^{-1} per a la majoria dels compostos, encara que en casos com la benzoilecgonina, la cocaïna i l'etil sulfat s'aconsegueixen valors de μg L^{-1}. En els efluents i en les aigües superficials del riu Túria es detecten menys drogues i a més baixes concentracions que en els influents. Els sòlids en suspensió i els fangs deshidratats de les estaciones depuradores contenen compostos cocaínics i opiacis a concentracions de ng g^{-1}.

Tercer. Entre els biomarcadors humans específics, les estimacions de la població realitzades amb les càrregues diàries de cafeïna, cotinina, i en una estació depuradora també amb l'àcid 5-hidroxiindolacètic van ser les més precises i comparables respecte a les dades del cens i els paràmetres hidroquímics. L'ús de biomarcadors per estimar la població ha de ser avaluat amb precaució, ja que en casos especials com la presència de fàbriques d'embotellat de begudes de cola, poden distorsionar els resultats obtinguts a partir de l'ús de la cafeïna com a biomarcador. No obstant açò, poden ser una eina inavaluable per reduir la incertesa en la grandària de la població servida per les estaciones depuradores d'aigües residuals.

Quart. L'aplicació de *l'epidemiologia de claveguera* en l'estudi realitzat durant diversos anys en tres estaciones depuradores que proveeixen a València i la seua àrea metropolitana, mostra que les drogues més consumides són cànnabis i cocaïna seguides de ketamina, amfetamina, metamfetamina i èxtasi. A més, posa de manifest que el consum d'alcohol durant la festivitat de les Falles s'incrementa fins a un 400 per cent respecte a una setmana considerada normal.

Cinquè. Les drogues seleccionades són eliminades en les estaciones depuradores amb una eficàcia superior al 95%, excepte l'èxtasi i la ketamina que són recalcitrants i difícilment s'eliminen. Els resultats obtinguts mostren que l'eficàcia d'eliminació varia lleugerament segons el tractament de les depuradores. Pinedo II, amb tractament terciari, mostra una major eliminació de les drogues seleccionades que les depuradores sense aquest tractament com Pinedo I i Quart-Benàger. Malgrat aquest fet, per als compostos recalcitrants seria convenient buscar tractaments alternatius.

Sisè. Existeix una gran dificultat per establir la relació entre les concentracions de les drogues d'abús en el medi ambient i la pressió humana en la conca del riu Túria, a causa de la presència esporàdica, el caràcter pseudopersistent i la complexa degradació d'aquests compostos. Tanmateix, l'aplicació del Sistema d'Informació Geogràfica, mostra qualitativament que la presència d'aquests contaminants està lligada a grans poblacions amb més de 10000 habitants.

Setè. Els microcosms que simulen el riu revelen que la degradació de la fluoxetina es duu a terme a través de processos fotoquímics i microbians, sent els fotoquímics els més significatius. Els processos microbians en aigües superficials mostren una lleugera enantioselectivitat afavorint la persistència del enantiòmer (*R*). En fangs actius on es van estudiar els processos metabòlics microbians, es va observar una estereoselectivitat pronunciada que

va afavorir la degradació de la (*S*)-fluoxetina i la formació de (*S*)-norfluoxetina. Aquests resultats posen de manifest la importància de l'estudi dels enantiòmers.

Vuitè. Els quocients de risc calculats a tres nivells tròfics per a les drogues d'abús detectades en els efluents de les depuradores índica un risc agut mitjà per a l'èxtasi, baix per a la ketamina i sense risc per a la benzoilecgonina. Els estudis *in vivo* de toxicitat aguda realitzats per als enantiòmers de la fluoxetina i la norfluoxetina en organismes aquàtics, indiquen que no existeix enantioselectivitat en la resposta tòxica de *Daphnia magna* però sí que s'observa en el cas de *Tetrahymena thermophila*, sent 30 vegades superior la toxicitat de la (R)-fluoxetina que la del enantiòmer (S). Les concentracions de compostos detectats en el riu Túria no suposen un risc. Les drogues d'abús són pseudopersistents i estan subjectes a fenòmens de degradació complexos en el medi ambient, per la qual cosa una exposició crònica a aquests compostos podria produir efectes a llarg termini.

Novè. Els estudis realitzats per avaluar rutes d'exposició alternativa als derivats del cànnabis a través dels aliments demostren que, el contingut de tetrahidrocannabinol en les llavors de cànem utilitzades com a pinso per a animals és inferior al marcat per la EFSA. El tetrahidrocannabinol es detecta en fórmules de creixement en concentracions de ng L^{-1}, però no es detecta en altres tipus de llet, ni en fetges de vaca. No es detecten metabòlits del tetrahidrocannabinol en cap de les matrius alimentàries analitzades. Encara que les concentracions detectades no presenten perill per als éssers humans, el mètode analític desenvolupat pot ser una pedra angular per garantir la innocuïtat dels aliments.

CONCLUSIONES

De acuerdo a los objetivos de la presente Tesis Doctoral, la investigación llevada a cabo y los resultados descritos en los capítulos anteriores, se puede concluir:

Primero. La cromatografía líquida de ultra alta resolución acoplada a la espectrometría de masas en tándem, utilizando un triple cuadrupolo, es selectiva, sensible, fiable y eficaz para la detección de las drogas de abuso seleccionadas en matrices complejas. Adicionalmente, los espectrómetros de masas de alta resolución, como el sistema híbrido cuadrupolo tiempo de vuelo, no sólo determinan las drogas seleccionadas, sino que también identifican un gran número de otros compuestos no seleccionados, metabolitos y productos de degradación presentes en muestras de aguas residuales y superficiales.

Segundo. La aplicación de las metodologías analíticas desarrolladas muestra que, en los influentes de las estaciones depuradoras de aguas residuales, se detectaron concentraciones de ng L^{-1} para la mayoría de los compuestos, aunque en casos como la benzoilecgonina, la cocaína y el etil sulfato se alcanzan valores de μg L^{-1}. En los efluentes y en las aguas superficiales del río Turia se detectan menos drogas y a más bajas concentraciones que en los influentes. Los sólidos en suspensión y los lodos deshidratados de las estaciones depuradoras contienen compuestos cocaínicos y opiáceos a concentraciones de ng g^{-1}.

Tercero. Entre los biomarcadores humanos específicos, las estimaciones de la población realizadas con las cargas diarias de cafeína, cotinina, y en una estación depuradora también con el ácido 5-hidroxiindolacético fueron las más precisas y comparables respecto a los datos del censo y los parámetros hidroquímicos. El uso de biomarcadores para estimar la población debe ser evaluado con precaución, ya que en casos especiales como la presencia de fábricas de embotellado de bebidas de cola, pueden distorsionar los resultados

obtenidos a partir del uso de la cafeína como biomarcador. Sin embargo, pueden ser una herramienta invaluable para reducir la incertidumbre en el tamaño de la población servida por las estaciones depuradoras de aguas residuales.

Cuarto. La aplicación de la *epidemiología de alcantarilla* en el estudio realizado durante varios años en tres estaciones depuradoras que abastecen a Valencia y su área metropolitana, muestra que las drogas más consumidas son cannabis y cocaína seguidos de ketamina, anfetamina, metanfetamina y éxtasis. Además, pone de manifiesto que el consumo de alcohol durante la festividad de las Fallas se incrementa hasta un 400 por ciento respecto a una semana considerada normal.

Quinto. Las drogas seleccionadas son eliminadas en las estaciones depuradoras con una eficacia superior al 95%, excepto el éxtasis y la ketamina que son recalcitrantes y apenas se eliminan. Los resultados obtenidos muestran que la eficacia de eliminación varía ligeramente según el tratamiento de las depuradoras. Pinedo II, con tratamiento terciario, muestra una mayor eliminación de las drogas seleccionadas que las depuradoras sin este tratamiento como Pinedo I y Quart-Benáger. Sin embargo, para los compuestos recalcitrantes sería conveniente buscar tratamientos alternativos.

Sexto. Existe una gran dificultad para establecer la relación entre las concentraciones de las drogas de abuso en el medioambiente y la presión humana en la cuenca del río Turia, debido a la presencia esporádica, el carácter pseudopersistente y la compleja degradación de estos compuestos. Sin embargo, la aplicación del Sistema de Información Geográfica, muestra cualitativamente que la presencia de estos contaminantes está ligada a grandes poblaciones con más de 10000 habitantes.

Séptimo. Los microcosmos que simulan el río revelan que la degradación de la fluoxetina se lleva a cabo a través de procesos fotoquímicos y microbianos,

siendo los fotoquímicos los más significativos. Los procesos microbianos en aguas superficiales muestran una ligera enantioselectividad favoreciendo la persistencia del enantiómero (*R*). En lodos activos donde se estudiaron los procesos metabólicos microbianos, se observó una estereoselectividad pronunciada que favoreció la degradación de la (*S*)-fluoxetina y la formación de (*S*)-norfluoxetina. Estos resultados ponen de manifiesto la importancia del estudio quiral de los enantiómeros.

Octavo. Los cocientes de riesgo calculados a tres niveles tróficos para las drogas de abuso detectadas en los efluentes de las depuradoras índica un riesgo agudo medio para el éxtasis, bajo para la ketamina y sin riesgo para la benzoilecgonina. Los estudios *in vivo* de toxicidad aguda realizados para los enantiómeros de la fluoxetina y la norfluoxetina en organismos acuáticos, indican que no existe enantioselectividad en la respuesta tóxica de *Daphnia magna* pero sí que se observa en el caso de *Tetrahymena thermophila*, siendo 30 veces superior la toxicidad de la (*R*)-fluoxetina que la del enantiómero (*S*). Las concentraciones de compuestos detectados en el río Turia no suponen un riesgo. Las drogas de abuso son pseudopersistentes y están sujetas a fenómenos de degradación complejos en el medioambiente, por lo que una exposición crónica a estos compuestos podría producir efectos a largo plazo.

Noveno. Los estudios realizados para evaluar rutas de exposición alternativa a los derivados del cannabis a través de los alimentos demuestran que, el contenido de tetrahidrocannabinol en las semillas de cáñamo utilizadas como pienso para animales es inferior al marcado por la EFSA. El tetrahidrocannabinol se detecta en fórmulas de crecimiento en concentraciones de ng L^{-1}, pero no se detecta en otros tipos de leche, ni en hígados de vaca. No se detectan metabolitos del tetrahidrocannabinol en ninguna de las matrices alimentarias analizadas. Aunque las concentraciones detectadas no presentan peligro para los seres humanos, el método analítico desarrollado puede ser una piedra angular para garantizar la inocuidad de los alimentos.

CONCLUSIONS

According to the objectives of this Doctoral Thesis, the research carried out and the results described in the previous chapters, the conclusions were:

First. Ultra-high performance liquid chromatography tandem mass spectrometry, using triple quadrupole, is selective, reliable, and effective and presents high sensitivity for the systematic detection of selected drugs of abuse in complex matrices. Furthermore, ultra-high performance mass spectrometry using an hybrid system quadrupole time-of-flight achieved the determination of the selected drugs and suspect screening of several compounds, metabolites and degradation products using wide-scope analysis of wastewater and surface waters.

Second. The application of the developed analytical methods shows that the detected concentration of most compounds in wastewater treatment plants influent were at ng L^{-1}, although in cases such as benzoylecgonine, cocaine and ethyl sulfate reached values of μg L^{-1}. In effluents and surface waters of Turia River, less drugs at lower concentrations than in influents were detected. Suspended solids and dehydrated sludge from wastewater treatment plants included cocainics and opioids compounds at concentrations of ng g^{-1}.

Third. Among the human specific biomarkers, population estimations made with daily loads of caffeine, cotinine, and in one wastewater treatment plant with 5-hydroxyindolacetic acid, were the most accurate and comparable to the census data and hydrochemical parameters. The use of biomarkers to estimate the population should be evaluated very carefully, because even in the case of caffeine, special cases as the presence of cola soft-drink bottling factories can distort the results. However, they can be an invaluable tool to reduce uncertainty in population size served by the wastewater treatment plants.

Fourth. The implementation of *sewage epidemiology* in the study carried out for several years in three wastewater treatment plant that supply Valencia and its

metropolitan area, shows that the most consumed drugs were cannabis and cocaine followed by ketamine, amphetamine, methamphetamine and ecstasy. Furthermore, the consumption of alcohol determined during Fallas festivity increased up to 400 percent with respect to an ordinary week.

Fifth. The selected drugs are eliminated in wastewater treatment plants with an efficiency above than 95%, except ecstasy and ketamine that are recalcitrant and hardly eliminated. The results show that the elimination efficiency varies slightly depending on the treatment of each plant. Pinedo II, with tertiary treatment, shows a greater elimination of the selected drugs than the treatment plants without this treatment such as Pinedo I and Quart-Benager. However, for recalcitrant compounds it would be desirable to seek alternative treatments.

Sixth. There is a great difficulty in establishing the connection between concentrations of drugs of abuse in the environment and human pressure in the Turia River basin due to the sporadic presence, pseudo-persistent character and the complex degradation of drugs. However, the application of the Geographic Information System shows in a qualitatively way, that the presence of these pollutants is linked to large populations with more than 10000 inhabitants.

Seventh. The river simulating microcosms revealed that fluoxetine degradation takes place via photochemical and microbial processes, being the photochemical ones the most significant. Microbial processes in surface waters revealed a slight enantioselectivity towards the persistence of (*R*)-enantiomer. In activated sludge simulating microcosms, microbial metabolic processes were studied. A pronounced stereoselective favouring the degradation of (*S*)-fluoxetine and formation of (*S*)-norfluoxetine were observed. These results highlight the importance of the study of chirality and enantiomers.

Eighth. The calculated risk quotients at three trophic levels for detected drugs of abuse in effluents of wastewater treatment plants indicate a medium short-term risk for ecstasy, low for ketamine and no risk for benzoylecgonine. Regarding *in vivo* acute toxicity tests performed for enantiomers of fluoxetine and norfluoxetine for aquatic organisms, there is no significant enantioselectivity in the toxic response from *Daphnia magna* to both fluoxetine and norfluoxetine, a strong enantiomer-dependent toxicity is observed in the case of *Tetrahymena thermophila*, being (*R*)-fluoxetine 30x higher than (*S*)-enantiomer. Compounds detected in Turia River do not seem to pose acute risk. Drugs of abuse are pseudo-persistent and suffer complex degradation process in the environment, so chronic exposure to these compounds could produce undetermined long-term toxicity effects.

Ninth. The studies carried out to evaluate alternative routes of exposure to cannabis derivatives through food matrices show that the tetrahydrocannabinol content in hemp seeds used as animal feed is less than that marked by EFSA. Tetrahydrocannabinol is detected in infant formulas at concentrations of ng L^{-1}, but is not detected in other types of milk, or cow livers. No metabolites of tetrahydrocannabinol are detected in any of the analyzed food matrices. Although the detected concentrations do not present hazard to humans, the analytical developed method can be a cornerstone for ensuring food safety.

ANEXOS

ÍNDICE DE TABLAS

Capítulo 1

Capítulo 2

La nomenclatura "EPIX" significa: día de muestreo del influente de Pinedo I en el mes en el cual se recogieron las muestras, "n.d." se refiere a los compuestos que no se detectaron en las muestras

Tabla S2.3 Drogas detectadas tras el análisis de los sólidos en suspensión de los influentes de la WWTP de Pinedo II. Las muestras se recogieron entre el 04/03/2014 y el 20/03/2014. Los resultados se muestran en ng g^{-1} (ppb). La nomenclatura "EPIIX" significa: día de muestreo del influente de Pinedo II en el mes en el cual se recogieron las muestras, "n.d." se refiere a los compuestos que no se detectaron en las muestras

Tabla S2.4 Drogas detectadas tras el análisis de los sólidos en suspensión de los influentes de la WWTP de Quart-Benáger. Las muestras se recogieron entre el 04/03/2014 y el 20/03/2014. Los resultados se muestran en ng g^{-1} (ppb). La nomenclatura "EQBX" significa: día de muestreo del influente de Quart Benáger en el mes en el cual se recogieron las muestras, "n.d." se refiere a los compuestos que no se detectaron en las muestras

Tabla S2.5 Drogas detectadas tras el análisis de los lodos activos de la WWTP de Pinedo I y Pinedo II. Las muestras se recogieron entre el 04/03/2014 y el 20/03/2014. Los resultados se muestran en ng g^{-1} (ppb). La nomenclatura "LPX" significa: día de muestreo de los lodos activos de Pinedo en el mes en el cual se recogieron las muestras, "n.d." se refiere a los compuestos que no se detectaron en las muestras

Tabla S2.6 Drogas detectadas tras el análisis de los lodos activos de la WWTP de Quart Benáger. Las muestras se recogieron entre el 04/03/2014 y el 20/03/2014. Los resultados se muestran en ng g^{-1} (ppb). La nomenclatura "LQBX" significa: día de muestreo de los lodos activos de Quart Benáger en el mes en el cual se recogieron las muestras, "n.d." se refiere a los compuestos que no se detectaron en las muestras, "w.s." significa "sin muestra"

Tabla S2.7 Drogas detectadas tras el análisis de los sedimentos del río Turia. Los resultados se muestran en ng g^{-1} (ppb). La nomenclatura "ESEDX" significa: sedimento y referencia de la muestra, "n.d." se refiere a los compuestos que no se detectaron en las muestras

Publicación científica 3. *Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer*

Tabla 3.1 Recuperaciones absolutas (100 ng L^{-1}), efecto matriz (n=5) y LCLs del método completo para las diferentes matrices analizadas

Tabla 3.2 Comparación de los resultados cuantitativos obtenidos usando ABSciexTOF™ 5600 (QqTOF) y el triple cuadrupolo (QqQ) en muestras de influentes, efluentes y aguas de río

ÍNDICE DE FIGURAS

Capítulo 1

Capítulo 2

Publicación científica 3. *Analysis of psychoactive substances in water by information dependent acquisition on a hybrid quadrupole time-of-flight mass spectrometer*

Publicación científica 4. *Universal method to determine acidic licit and illicit drugs and personal care products in water by liquid chromatography quadrupole time-of-flight*

Publicación científica 5. *Occurrence and removal of drugs of abuse in Wastewater Treatment Plants of Valencia (Spain)*

Figura S9.6 Confirmación cualitativa de los tiempos de retención de los enantiómeros puros. En orden de arriba abajo *R*-FL MRM1 RT = 39.44; *R*-FL MRM2 RT = 39.41; *S*-FL MRM1 RT = 36.57; *S*-FL MRM2 = 36.50

Publicación científica 10. *Analysis of cannabinoids by liquid chromatography-mass spectrometry in milk, liver and hemp seed to ensure food safety*

Figura 10.1 Comparación de las recuperaciones obtenidas usando diferentes (A) cartuchos SPE (B) volúmenes de elución de metanol (MeOH) (C) volúmenes de redisolución de MeOH y MeOH + 0.1 % ácido fórmico (FA). Nivel de concentración de estándares a 500 μg L^{-1} en leche

Figura 10.2 ME obtenidos usando curvas de calibración con IS en semillas de cáñamo, leche e hígado fortificados a niveles de concentración entre 10 y 750 μg L^{-1}

Figura 10.3 Cromatogramas de (A) muestras de leche positivas en THC y (B) muestras de hígado con supuestos metabolitos del THC

Figura S10.1 Separación cromatográfica del THC, THC-COOH y THC-OH obtenidos para soluciones estándar a 100 μg L^{-1}. Fase móvil compuesta por (A) agua desionizada y (B) metanol, ambos con 10 mM formato amónico

Figure S10.2 Condiciones del gradiente de elución a 100 μg L^{-1} de la solución de los compuestos estándar: 0 min – 70% B, 5 min – 95% B para a) fase móvil compuesta por (A) agua desionizada y (B) metanol, ambos con 0.1 % ácido fórmico y b) (A) agua desionizada y (B) metanol, ambos con 10 mM de formato amónico

Figura S10.3 Linealidad en matrices complejas (leche e hígado) de THC, THC-COOH y THC-OH

Figura S10.4 Cromatograma del THC detectado en una muestra de semillas de cáñamo

ÍNDICE DE ABREVIATURAS

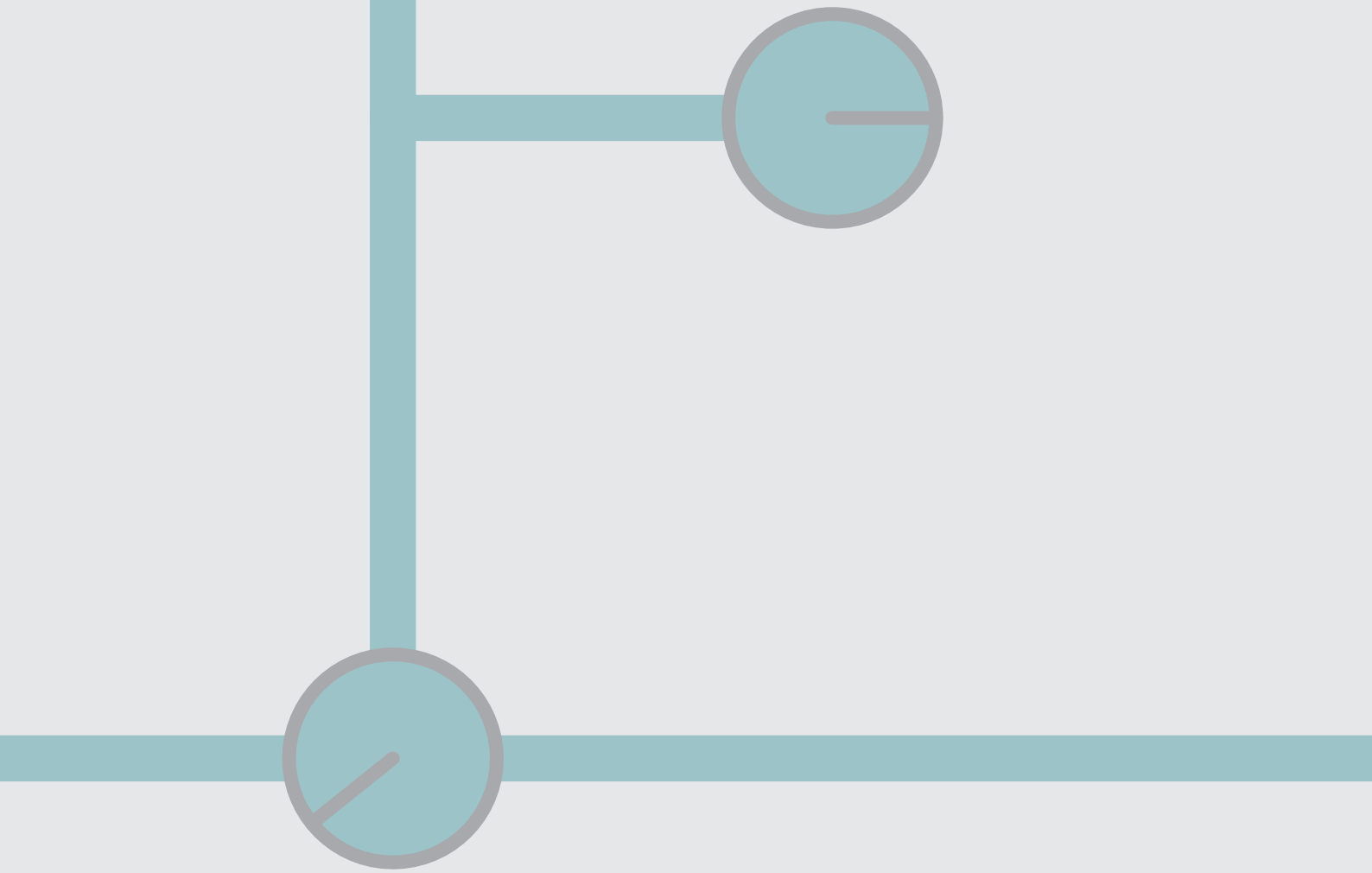
En el índice de las abreviaturas se presentan aquellas más representativas de la presente Tesis Doctoral. En cada una de las publicaciones científicas se detallan todas las abreviaturas utilizadas.

2C-B	4-Bromo-2,5-dimetoxifenetilamina - 4-Bromo -2,5- dimethoxyphenethyl-amine
4-AcO-DIPT	4-acetoxy-N,N-dimetiltriptamina – 4-acetoxy-N,N-dimethyltryptamine
4-MeO-PCP	4-metoxifenciclidina – 4-methoxyphencyclidine
4-MePPP	4-metil-α-pirrolidinopropiofenona – 4-methyl-α-pyrrolidinopropiophenone
4'-MePHP	4-metil-α-pirrolidinohexafenone – 4-methyl-α-pyrrolidinohexaphenone
6-MAM	6-acetilmorfina – 6-acetylmorphine
αPVP	α- pirrolidinopentiofenona – α- pyrrolidinopentiophenone
AAC	Acetato amónico – Ammonium acetate
AMP	Anfetemina – Amphetamine
BE/BECG	Benzoilecgonina – Benzoylecgonine
bk-MMBDB	Dibutilona – Dibutylone
BOD	Demanda biológica de oxígeno – Biological oxygen demand
BUF	Bufotenina – Bufotenine
CCα	Límite de decisión – Decision limit
CCβ	Capacidad de detección – Detection capability
CE	Energía de colisión – Collision energy
COC	Cocaína – Cocaine
COCET	Cocaetileno – Cocaethylene
COD/	
CODE	Codeína – Codeine
COD	Demanda química de oxígeno - Chemical oxygen demand
Cond	Conductividad – Conductivity
DAR	Reactor en oscuridad y abiótico – Dark abiotic reactor
DBR	Reactor en oscuridad y biótico – Dark biotic reactor
DDA	Adquisición de datos dependiente – Data dependent acquisition
DIA/IDA	Adquisición de datos independiente – Data independent acquisition
DO	Oxígeno disuelto – Dissolved oxygen
EC50	Concentración media del efecto máximo – Half maximal effective concentration
ECME	Ecgonina metil ester – Ecgonine methyl ester
EDDP	2-etilideno-1,5-dimetil-3,3-difenilpirrolidina – 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

EFSA	Autoridad Europea de Seguridad Alimentaria – European Food Safety Authority
EMCDDA	Observatorio Europeo de las Drogas y Toxicomanías – European Monitoring Centre for Drug and Drug Addiction
EPH	Efedrina – Ephedrine
EPHED	Efedrona – Ephedrone
ERA	Evaluación del riesgo ambiental – Environmental risk assessment
ESI	Ionización por electorspray – Electospray ionization
ETAMINE	Etilanfetamina – Ethylamphetamine
EtOH	Etanol – Ethanol
ETONE	Etilona – Ethylone
FL	Fluoxetina – Fluoxetine
GIS	Sistema de información geográfica – Geographical information system
HER	Heroína – Heroin
HLB	Equilibrio hidrofílico-lipofílico – hydrophilic-lipophilic balanced
HR	Alta resolución – High resolution
IDL	Límite de detección instrumental – Instrumental detection limit
IQL	Límite de cuantificación instrumental – Instrumental quantification limit
KET	Ketamina – Ketamine
LAR	Reactor luz y abiótico – Light abiotic reactor
LBR	Reactor luz y biótico – Light biotic reactor
LCL	Nivel de calibración más bajo – Lowest calibration level
LC-MS	Cromatografía líquida espectrometría de masa – Liquid chromatography mass spectrometry
LLE	Liquid-liquid extraction – Extracción líquido-líquido
LOD	Límite de detección – Limit of detection
LOQ	Límite de cuantificación – Limit of quantification
LR	Low resolution – Baja resolución
MAMP	Metanfetamina – Methamphetamine
MBDB	N-Metil-1-(3,4-metilendioxifenil)-2-butanamida – N-Methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
mCPP	1-(3-clorofenil)piperazina – 1-(3-chlorophenil)piperazine
MDA	3,4-metilendioxianfetamina – 3,4-methylendioxyamphetamine
MDEA	3,4-metilenedioxietilanfetamina – 3,4-methylenedioxyethylamphetamine
MDMA	3,4-metilenedioximetanfetamina 3,4-methylenedioxymethamphetamine
MDPPP	3',4'-Metilenedioxi-α-pirrolidinapropiofenona –

	3',4'-Methylenedioxy-α-pyrrolidinopropiophenone
MDPV	Metilenedioxipirovalerona – Methylenedioxypyrovalerone
ME	Efecto matriz – Matrix effect
MeOH	Metanol – Methanol
MEP	Mefedrona – Mephedrone
MET	Metadona – Methadone
METONE	Metilona – Methylone
MEPHEN	Metilfenidato – Methylphenidate
MOR	Morfina – Morphine
MPBP	4-metil-α-pirrolidinobutiofenona – 4-methyl-α-pyrrolidinobutiophenone
MRM	Monitorización de reacción múltiple – Multiple reaction monitoring
N	Nitrógeno – Nitrogen
NAPH	Nafirona – Naphyrone
NFL	Norfluxetina – Norfluoxetine
NPS	Nuevas sustancias psicoactivas – New psychoactive substances
OD	Densidad óptica – Optical density
P	Fósforo – Phosphorous
PCA	Análisis de componentes principales – Principal component analysis
PI	Ionización positiva – Positive ionization
PMA	4-metoxianfetamina – 4-methoxyamphetamine
PPP	α-pirrolidinopropiofenona – α-pyrrolidinopropiophenone
QqQ	Espectrómetro de masas triple cuadrupolo – Triple quadrupole mass spectrometer
QqTOF	Cuadrupolo tiempo de vuelo – Quadrupole time-of-flight
QTRAP	Cuadrupolo lineal trampa de iones – Quadrupole linear ion-trap
Res	Resistencia – Resistivity
RQ	Coeficiente de riesgo – Risk quotient
R_s	Resolución de separación enantiomérica – Resolution of separation of enantiomers
RSD	Desviación estándar relativa – Relative standar deviation
SLE	Extracción sólido-líquido – Solid-liquid extraction
SCORE	Grupo de análisis de aguas residuales de Europa – Sewage analysis CORe groupe Europe
SPE	Extracción en fase sólida – Solid phase extraction
SRM	Monitorización de reacción selective – Selected reaction monitoring
TDS	Sólidos totales disueltos – Total disolved solids
TFMPP	1-(3-trifluorometilfenil)piperazina – 1-(3-trifluoromethylphenyl)piperazine
THC	Δ9-Tetrahidrocannabinol – Δ9-Tetrahydrocannabinol

THC-COOH	11-nor-9-carboxi-Δ9-tetrahidrocannabinol – 11-nor-9-carboxy-Δ9-tetrahydrocannabinol
THC-OH	11-hidroxi- Δ9- tetrahidrocannabinol – 11-hidroxy- Δ9- tetrahydro-cannabinol
TIC	Cromatograma de iones totales – Total ion cromatogram
TPs	Productos de transformación – Transformation products
t_R/R_t	Tiempos de retención – Retention time
UAE	Extracción asistida por ultrasonidos – Ultrasound assisted extraction
UHPLC	Cromatografía líquida de alta resolución – Ultra-high performance liquid chromatography
WBE	Epidemiología basada en aguas residuales – Wastewater based epidemiology
WWTP	Estaciones depuradoras de aguas residuales – Wastewater treatment plant



CIDE Centro de Investigaciones sobre Desertificación

CSIC
Consejo Superior de Investigaciones Científicas

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Ministerio de Economía, Industria y Competitividad

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