UNIVERSITAT JAUME·I

THE INVESTIGATION OF ILLICIT DRUGS AND THEIR METABOLITES IN WATER BY LIQUID CHROMATOGRAPHY COUPLED TO LOW AND HIGH RESOLUTION MASS SPECTROMETRY

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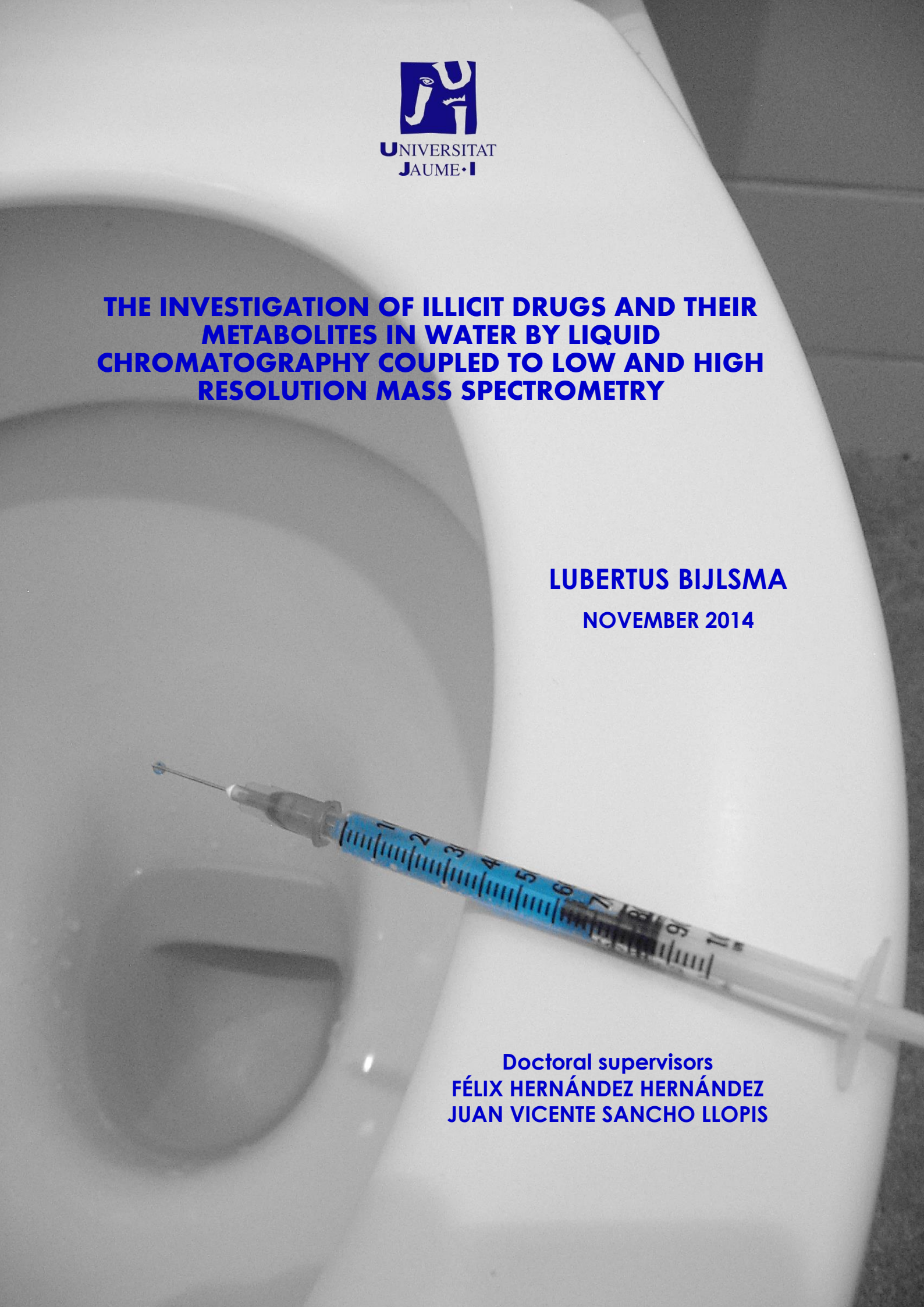

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THE INVESTIGATION OF ILLICIT DRUGS AND THEIR METABOLITES IN WATER BY LIQUID CHROMATOGRAPHY COUPLED TO LOW AND HIGH RESOLUTION MASS SPECTROMETRY

Doctoral Thesis

Lubertus Bijlsma

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Certifican: que la Tesis Doctoral "The investigation of illicit drugs and their metabolites in water by liquid chromatography coupled to low and high resolution mass spectrometry" ha sido desarrollada bajo su dirección, en el Instituto Universitario de Plaguicidas y Aguas, Departamento de Química Física y Analítica de la Universitat Jaume I de Castellón, por **Lubertus Bijlsma**.

Lo que certificamos para los efectos oportunos en Castellón de la Plana, a 21 de julio de 2014.

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This thesis has been carried out, and will consequently be defended, in order to obtain the title "Doctor internacional". The PhD candidate fulfilled the requirements for an international thesis as regulated within the framework of the Real Decreto 99/2011, "Normativa de los estudios de doctorado", at the University Jaume I.

Lubertus Bijlsma performed two secondments of a total time of three months. The first secondment has been carried out at the *National Institute for Public Health and the Environment "RIVM"* in Bilthoven, the Netherlands, from the 29th of October till the 30th of November, 2009. The work entitled "Monitoring party drugs in drinking water sources" has been performed under the supervison of Ir. J.F.M Versteegh and Dr. R.A. Baumann. The stay in this research centre allowed the candidate to increase his knowledge on analytical aspects related to the occurrence of illicit drugs and their metabolites in surface water and drinking water produced from those waters. The second secondment was carried out at the *KWR Watercycle Research Institute* in Nieuwegein, the Netherlands, from the 15th of February till the 16th of April, 2010. The work entitled "Determination of drugs of abuse and their metabolites in urban wastewaters" was performed under the supervison of Prof. Dr. W.P. de Voogt. The stay in this research centre allowed the candidate to increase his knowledge on analytical aspects of High Resolution Mass Spectrometry to investigate the presence of illicit drugs in wastewater as well as to study the removal efficiency of each compound by the wastewater treatment plants.

The work performed in this thesis was part of the activities described in the projects (Group of Excellence Prometeo 2009/054; Collaborative Research on Environment and Food Safety ISIC/2012/016) of the Generalitat Valenciana.

Prior to the defense of the Doctoral thesis, this work was independently evaluated by two external reviewers, Dr. E.A Hogendoorn *(Centre for Safety of Substances and Products, National Institute for Public Health and the Environment,* Bilthoven, the Netherlands*)* and Dr. L. Barron *(Analytical & Environmental Sciences Division, King's College London*, London, United Kingdom).

Voor Sofia

Summary

Investigation of illicit drugs (IDs) in water has become a prominent topic over the last few years. IDs may enter the sewage system, unaltered or as metabolites, after consumption and excretion. Treatment processes commonly applied by wastewater treatment plants (WWTPs) are often insufficient and some IDs and/or transformation products (TPs) are continuously released into the aquatic environment. The determination of illicit drugs and their metabolites in the water cycle can contribute to the understanding of the potential impact of these compounds on the aquatic ecosystem, but data from analysis can also provide information on drug use and trends. Hence, sophisticated analytical methodologies are required to obtain accurate concentration data on these compounds in the aquatic environment.

In this doctoral thesis, the potential of modern analytical techniques, based on hyphenated liquid chromatography tandem mass spectrometry (LC-MS/MS) with triple quadrupole (QqQ), hybrid quadrupole time-of-flight (QTOF) and linear ion trap Orbitrap (LTQ-Orbitrap) mass analyzers, has been investigated for the determination of IDs and their metabolites/transformation products in different types of water samples.

The work has been structured in five parts. Nine scientific articles, and one book chapter written by invitation, have resulted from the data obtained in this thesis. In the first part, the potential of UHPLC-MS/MS with triple quadrupole has been explored for the quantitative determination of frequently consumed IDs and metabolites in water. Two multi-residue methods based on a pre-treatment step by off-line solid-phase extraction (SPE) followed by UHPLC-MS/MS determination have been developed. In both methods, compounds belonging to different classes of IDs, with diverse physico-chemical properties could be quantified and properly identified in a single analysis. In the first method, 11 basic/acidic compounds belonging to three classes of IDs (amphetamines, cocaine and cannabis) were selected on the basis of their wide consumption in the Mediterranean area. Later, the method was updated taking advantage of the availability of a state-of-the-art UHPLC-MS/MS instrument recently acquired, resulting in the method presented in the second work. In addition, the scope of the second method was increased, including several opioids and ketamine.

Selection of these compounds was made on the basis of data obtained in a European-wide monitoring study, where, for example, ketamine was occasionally detected in wastewaters samples. A detailed study on minimization of matrix effects has been carried out, evaluating their impact on the chromatographic performance and sensitivity. Both methods were fully validated and applied to the analysis of urban wastewaters from WWTPs located in the province of Castellón, Spain. Some of these results have formed a part of a European-wide monitoring, supported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), where our laboratory was one of the participating centers. Cocaine and cannabis were the most frequently detected IDs. Moreover, the results indicated an increase of cocaine use during weekends and an increase of most other drugs during an important music festival. Concentration data from influent and effluent wastewater allowed estimating the removal efficiency of each compound by the WWTPs, which was generally satisfactory for low concentration levels found in influent wastewaters. However, the removal of IDs decreased during the week of the rock festival.

The second part consists of a book chapter and two scientific articles and focuses on the potential of LC coupled to High Resolution Mass Spectrometry (HRMS) to investigate the presence of IDs in wastewater. The versatility of LC, together with the high mass resolving power and high mass accuracy of HRMS, and the possibility of performing MS/MS or pseudo MS/MS experiments, allowed the detection and identification of IDs and their metabolites with the required selectivity and sensitivity. Firstly, the potential of UHPLC-QTOF MS has been explored for rapid qualitative screening of 76 IDs, prescription drugs with potential for abuse and some of their metabolites in wastewater samples collected in Spain. Analyses were performed under MS^E acquisition mode, which involves the simultaneous acquisition of two functions at different collision energies. This allows obtaining accurate mass data of both protonated molecules (low collision energy) and fragment ions (high collision energy) in a single run. The screening of suspects in influent and effluent wastewater samples allowed the detection and identification of ten compounds, of which five compounds (codeine, cotinine (metabolite of nicotine), ketamine, oxazepam and temazepam) could be tentatively identified without initially having the reference standards. The qualitative and

quantitative performance of an LTQ Orbitrap mass analyzer was also studied for 24 IDs and relevant metabolites in wastewater, using full-scan accurate mass data and simultaneous MS^n measurements. The latter, was generated in the LTQ and provided useful information of product ions. The method was validated, and Orbitrap MS proved to have satisfactory quantitative capabilities for the determination of IDs at sub-ppb levels. The methodology was applied to wastewater samples from the Netherlands. Several IDs such as MDMA, benzoylecgonine (BE), codeine and benzodiazepines could be identified and quantified. In addition, MS data provided by Orbitrap allowed retrospective investigation of ketamine metabolites in the samples.

The third part consists of two scientific articles where accurate mass spectra provided by LC-QTOF MS under MS/MS and MS^E modes allowed to investigate the fragmentation of several IDs and their metabolites by one side, and the elucidation of TPs of cocaine and its main metabolite BE in water by other side. In the first work, fragmentation pathways of several classes of IDs (amphetamines, cocaine, opioids, cannabinoids and ketamine) and related substances, including metabolites and deuterated analogues, have been investigated. Chemical structures of fragment ions were carefully proposed using the accurate mass data together with basic fragmentation concepts and rules. Our accurate mass data allowed confirming structures and fragmentation pathways previously reported making use of nominal mass data, but also gave new insights into amphetamine and amphetamine-type stimulants, the main metabolite of cannabis and opiates. In the second work, a study of TPs of cocaine and BE was performed. Cocaine was selected because it is highly consumed and high concentrations are commonly found in influent wastewaters. The possible formation of TPs during wastewater treatment processes or due to the exposure to natural elements in the environment (e.g. sunlight) must be taken into account as these TPs may be more persistent than, or exhibit similar toxicity than the parent compound. Several degradation experiments were carried out under laboratory-controlled conditions. The accurate mass data and previous knowledge of the fragmentation pathways of cocaine and BE were very helpful for the elucidation of several TPs. Various known metabolites / TPs could be confirmed and other unknown compounds could tentatively be identified, even though no reference standards were available. Up to sixteen and

ten TPs of cocaine and BE were found, respectively. Four non-previously reported TPs were detected in effluent wastewater and surface water samples.

The fourth part consists of three scientific articles, focusing on the occurrence of IDs in the aquatic environment. Water samples from Spain and the Netherlands were analyzed applying quantitative analytical methodologies developed in the first and second part of this thesis. The objective was to study the presence of the selected IDs and metabolites, as well as their possible removal by the WWTPs, and also their potential impact on the aquatic environment. Special emphasis has been given to quality assurance. Quantification and confirmation criteria were applied following European guidelines and quality control samples were analyzed in every sequence of sample analysis. In the first work, IDs were determined daily in both influent and effluent wastewater samples from three WWTPs located in the province of Castellón, Spain. Samples were collected in three one-week periods, including one week which coincided with an international music festival in Benicassim. Under normal circumstances, IDs concentrations were low and removal efficiencies were satisfactory. A notable increase in concentrations, however, was observed during the festival. This was also reflected by higher analyte levels in the effluents and higher loads to the aquatic environment. In the second work, 24 target drugs were investigated by LTQ Orbitrap MS in wastewater samples from five WWTPs located in the Netherlands, including the international airport of Amsterdam (Schiphol). Daily variances of drug loads and removal efficiencies were estimated for each drug and WWTP individually. The results contributed to a better insight on the presence of IDs in Dutch wastewaters, and showed a slightly different occurrence pattern in wastewaters from the airport. Removal efficiencies of WWTPs were generally satisfactory except for benzodiazepines and MDMA. In the third work, a wide screening of 34 IDs and their relevant metabolites in the Dutch watercylce was performed in a collaborative study where four laboratories participated. Urban wastewater (influent and effluent), surface water of the rivers Rhine and Meuse, and drinking water that are produced from those rivers were analysed. The results suggested that substantial fractions of the total load of IDs in the rivers Rhine and Meuse enter the Netherlands from abroad. For some compounds, loads appeared to increase downstream, which seemed to be caused by the contribution from Dutch

wastewater effluents. In finished drinking water only BE was detected in only one sample, and at a concentration below the limit of quantification. No toxicological relevance for environment and human health were expected from the data of this study. Nevertheless, further research with respect to possible long-term (chronic) effects on living organisms and possible effects of combined exposure to multiple compounds at low concentrations were recommended.

The fifth part does not formally form the core part of this doctoral thesis. Just a brief summary is presented, as it is worthwhile to mention the effort made to establish a European-wide network to monitor the presence of IDs in wastewater and to estimate the use of these compounds in the European cities under study. The PhD candidate has been closely involved in this network and his key role within the network was to give support on analytical issues. Advanced analytical techniques, commonly based on hyphenated chromatography mass spectrometry, are the cornerstone to obtain accurate concentration data on excretion products of IDs in sewage water. This is a clear example where modern analytical chemistry plays a key role in getting more knowledge on use and trends of illicit drugs in a population. Three collaborative articles and one report are included in this part, as illustrative examples of the above mentioned European collaboration. These articles highlight the differences in drug use in several areas of Europe and give a general overview of the analytical methodologies commonly applied in this field, as well as uncertainties associated with the sewage-based epidemiology approach.

Resumen

La investigación de drogas ilícitas (IDs) en aguas se ha convertido en tema de actualidad en los últimos años. Las IDs pueden entrar en las aguas residuales, inalteradas o como metabolitos, después de su consumo y excreción. Los procesos de depuración, comúnmente aplicados en las plantas de tratamiento de aguas residuales (WWTPs), son a menudo insuficientes y algunas IDs y/o sus productos de transformación (TPs) pueden ser transferidos continuamente a aguas medioambientales. La determinación de IDs y sus metabolitos en el ciclo del agua puede contribuir a entender el potente impacto de estos compuestos en el ecosistema acuático, así como proveer información sobre el consumo de drogas y sus tendencias. Por consiguiente, se requiere desarrollar metodologías analíticas sofisticadas para obtener datos de concentración exactos de estos compuestos en el medio acuático.

En esta tesis doctoral, se ha investigado el potencial de técnicas analíticas modernas basadas en el acoplamiento cromatografía líquida-espectrometría de masas en tándem (LC-MS/MS), con analizadores de triple cuadrupolo (QqQ), híbrido cuadrupolo-tiempo de vuelo (QTOF) y trampa de iones lineal Orbitrap (LTQ-Orbitrap), para la determinación de IDs y sus metabolitos/TPs en diferentes tipos de muestras de agua.

El trabajo se ha estructurado en cinco partes. De los datos obtenidos en esta tesis han resultado nueve artículos científicos y un capítulo de un libro, escrito por invitación.

En la primera parte, se ha explorado el potencial de la UHPLC-MS/MS, con analizador de triple cuadrupolo, para la determinación cuantitativa de las IDs más frecuentemente consumidas y sus metabolitos en agua. Se han desarrollado dos métodos multiresiduales basados en una etapa de pre-tratamiento de muestra mediante extracción en fase sólida (SPE) "*off-line*" y posterior determinación por UHPLC-MS/MS. En ambos métodos, se pudieron cuantificar e identificar en un solo análisis compuestos pertenecientes a diferentes clases de IDs, con propiedades físico-químicas diferentes. En el primer método se seleccionaron 11 compuestos ácido/base

pertenecientes a tres clases de IDs (anfetaminas, cocaína y cánnabis) en base a su elevado consumo en el área Mediterránea. Posteriormente, el método se actualizó tras la reciente adquisición de un instrumento UHPLC-MS/MS, resultando el método presentado en el segundo trabajo, en el cual se aumentó el espectro de aplicación a varios opiáceos y a la ketamina. La selección de estos compuestos se realizó de acuerdo con los datos obtenidos en estudios de control europeos donde, por ejemplo, la ketamina fue detectada, ocasionalmente, en muestras de agua residual. Se llevó a cabo un estudio detallado para la minimización del efecto matriz, evaluando su impacto en la determinación cromatográfica y en la sensibilidad. Ambos métodos fueron validados y, posteriormente, aplicados al análisis de aguas residuales urbanas de WWTPs situadas en la provincia de Castellón, España. Algunos de estos resultados forman parte del programa de control europeo, apoyado por el "*Observatorio Europeo de las Drogas y las Toxicomanías*" (EMCDDA), donde nuestro laboratorio es uno de los centros participantes. Las IDs más frecuentemente detectadas fueron la cocaína y el cannabis. Además, los resultados indicaron un aumento del consumo de la cocaína durante los fines de semana, así como un aumento de la mayoría de las otras drogas durante algún festival de música importante. Los datos de concentración obtenidos para las aguas residuales de entrada y salida permitieron estimar la eficiencia de las WWTPs en la eliminación de cada compuesto estudiado, la cual fue, generalmente, satisfactoria para los bajos niveles de concentración encontrados en las aguas residuales de entrada. Sin embargo, la eliminación de IDs disminuyó durante la semana del festival de rock.

La segunda parte resultó en un capítulo de libro y dos artículos científicos, focalizándose en el estudio del potencial de la LC acoplada a Espectrometría de Masas de Alta Resolución (HRMS) para investigar la presencia de IDs en aguas residuales. La versatilidad de la LC, junto con el alto poder de resolución del masas y la masa exacta de la HRMS, así como la posibilidad de realizar experiencias en modo MS/MS o pseudo MS/MS, permitió la detección e identificación de IDs y sus metabolitos con la selectividad y sensibilidad requerida. Inicialmente, se exploró el potencial de la técnica UHPLC-QTOF MS para un rápido "*screening*" cualitativo de 76 IDs, fármacos con potencial de abuso y algunos de sus metabolitos en muestras de agua residual

recogidas en España. Los análisis fueron llevados a cabo en modo MS^E, el cual implica la adquisición simultánea de dos funciones con energías de colisión diferentes. Esto permite obtener datos de masa exacta para las moléculas protonadas (baja energía de colisión) e iones fragmento (alta energía de colisión) en un solo análisis. El "*screening*" de muestras de agua residual de entrada y salida sospechosas de estar contaminadas permitió la detección e identificación de diez compuestos, de los cuales cinco analitos (codeína, cotinina (metabolito de la nicotina), ketamina, oxazepam y temazepam) pudieron ser provisionalmente identificados sin disponer inicialmente de patrones de referencia. El análisis cualitativo y cuantitativo mediante el analizador de masas LTQ Orbitrap fue también estudiado para 24 IDs y metabolitos relevantes en aguas residuales utilizando simultáneamente datos de masa exacta de "*full-scan*" y medidos en MS^n. Este último fue generado en el LTQ y facilitó información útil de iones producto. La validación del método demostró satisfactoriamente la capacidad cuantitativa para la determinación de IDs a niveles de sub-ppb. La metodología fue aplicada a muestras de aguas residuales de los Países Bajos. Varias IDs tales como MDMA, benzoilecgonina (BE), codeína y benzodiacepinas pudieron ser identificadas y cuantificadas. Además, los datos de MS obtenidos mediante el Orbitrap permitieron una investigación retrospectiva de los metabolitos de la ketamina en las muestras.

La tercera parte consistió en la elaboración de dos artículos científicos donde el espectro de masa exacta obtenido por LC-QTOF MS en modos MS/MS y MS^E permitió, por una parte, investigar la fragmentación de varios IDs y sus metabolitos, y por otra, la elucidación de TPs de la cocaína y su principal metabolito, la BE, en agua. En el primer trabajo, se estudiaron las vías de fragmentación de diferentes clases de IDs (anfetaminas, cocaína, opiáceos, cannabinoides y ketamina) y sustancias relacionadas, incluyendo metabolitos y análogos deuterados. Se propusieron estructuras químicas de iones fragmento utilizando los datos de masa exacta medida junto con los conceptos y reglas básicas de fragmentación. Nuestros datos de masa exacta permitieron confirmar estructuras y vías de fragmentación, previamente reportadas, haciendo uso de la masa nominal y al mismo tiempo, aportaron nuevos conocimientos para la anfetamina, estimulantes tipo-anfetamina, el principal

metabolito del cannabis y opiáceos. En el segundo trabajo, se llevó a cabo un estudio de los TPs de la cocaína y la BE. La cocaína se seleccionó por su alto consumo y las elevadas concentraciones comúnmente encontradas en aguas residuales de entrada. La posible formación de TPs tanto durante los procesos de tratamiento de aguas residuales como por la exposición a elementos naturales del medio ambiente (*p.e.* la luz solar) debe tenerse en cuenta ya que estos TPs pueden ser más persistentes o presentar una toxicidad similar al compuesto inicial. Se llevaron a cabo varios ensayos de degradación en el laboratorio bajo condiciones controladas. Los datos de masa exacta medida y el conocimiento previo sobre vías de fragmentación de la cocaína y la BE fueron de gran ayuda para la elucidación de diversos TPs. Varios metabolitos/TPs conocidos pudieron ser confirmados y otros compuestos desconocidos pudieron ser provisionalmente identificados, incluso sin disponer de los patrones de referencia. Se encontraron 16 TPs de la cocaína y 10 de la BE. Cuatro TPs, no reportados anteriormente, fueron detectados en muestras de agua residual de salida y en agua superficial.

La cuarta parte engloba tres artículos científicos focalizados en la formación de IDs en el medio acuático. Tanto las muestras de agua de España como las de los Países Bajos fueron analizadas mediante las metodologías analíticas cuantitativas desarrolladas en la primera y segunda parte de esta tesis. El objetivo fue el estudio de la presencia de IDs y metabolitos seleccionados, así como su posible eliminación en las WWTPs y su impacto potencial en el medio acuático. Se ha llevado a cabo con un especial énfasis el aseguramiento de la calidad. Los criterios de cuantificación y confirmación fueron aplicados siguiendo las guías europeas, y en cada secuencia de muestras se analizaron muestras de control de calidad (QCs). En el primer trabajo, las IDs se determinaron diariamente en muestras de agua residual, tanto de entrada como de salida, recogidas de tres WWTPs situadas en la provincia de Castellón, España. Se recogieron muestras de 24h en tres periodos semanales, incluyendo una semana en la cual se celebró un festival musical internacional en Benicassim. Bajo circunstancias normales, las concentraciones de IDs fueron bajas y las eficiencias en la eliminación fueron satisfactorias. Sin embargo, durante el festival se observó un notable aumento en las concentraciones. Esto también reflejó mayores niveles de los analitos en las

salidas y mayores cargas en el ambiente acuático. En el segundo trabajo, se investigó la presencia de 24 drogas "*target*" por LTQ Orbitrap MS en muestras de aguas residuales de cinco WWTPs situadas en los Países Bajos, incluyendo el aeropuerto internacional de Amsterdam (Schiphol). Se estimaron las variaciones diarias de cargas de droga y la eficiencia de cada WWTP para la eliminación de las drogas estudiadas de manera individual. Los resultados contribuyeron a una mejor comprensión de la presencia de IDs en las aguas residuales de los Países Bajos, revelando en las muestras del aeropuerto un modelo de comportamiento ligeramente diferente. Las eficiencias de las WWTPs en la eliminación fueron generalmente satisfactorias a excepción de las benodiazepinas y MDMA. En el tercer trabajo, se llevó a cabo un amplio "*screening*" de 34 IDs y sus metabolitos más relevantes en el ciclo del agua de los Países Bajos enmarcado en un estudio de colaboración con cuatro laboratorios participantes. Se analizaron muestras de agua residual urbana (entrada y salida), agua superficial de los ríos Rhine and Meuse, y agua potable producida de estos ríos. Los resultados sugirieron que importantes fracciones de la carga total de las IDs en los ríos Rhine and Meuse llegan a los Países Bajos del extranjero. Para algunos compuestos, las cargas parecían aumentar aguas abajo, lo cual podría estar causado por la contribución de las salidas neerlandesas de las aguas residuales. En el agua potable, únicamente se detectó BE en una muestra y a un nivel de concentración por debajo del límite de cuantificación. Con los datos obtenidos en este estudio, no se desprende ninguna relevancia toxicológica para el medio ambiente y la salud humana. No obstante, debería recomendarse una investigación futura basada en los posibles efectos a largo plazo (crónicos) en los organismos vivos y los posibles efectos en combinación con la exposición a múltiples compuestos a bajos niveles de concentración.

La quinta parte no es, en realidad, un trabajo que pertenezca formalmente a esta tesis doctoral. Se presenta únicamente como un breve resumen ya que merece la pena mencionar por el esfuerzo realizado para establecer una red europea que supervise la presencia de IDs en aguas residuales y estime el consumo de estos compuestos en las ciudades europeas investigadas. El autor de la presente tesis doctoral ha contribuido a esta red participando activamente en los temas analíticos. Las técnicas analíticas avanzadas, comúnmente basadas en acoplamiento cromatografía-espectrometría

de masas, son la base para obtener datos de concentraciones exactas en la excreción de los productos de IDs en las aguas residuales. Es un claro ejemplo donde la química analítica moderna juega un papel clave en la obtención de más conocimientos sobre el uso y las tendencias de las drogas ilícitas en la población. En esta parte se incluyen tres artículos de colaboración y un informe, como ejemplos ilustrativos de la colaboración europea mencionada. Estos artículos ponen de relieve las diferencias en el consumo de drogas en distintas zonas de Europa y dan una visión general de los métodos analíticos comúnmente aplicados en este campo, así como las incertidumbres asociadas a estudios epidemiológicos basados en los análisis de aguas residuales.

Samenvatting

Onderzoek naar de aanwezigheid van verslavende middelen (drugs) in water is de laatste jaren een belangrijk onderwerp geworden. Drugs kunnen na inname door uitscheiding onveranderd of als metaboliet in het rioleringsstelsel terecht komen. De processen die door afvalwaterzuiveringsinstallaties (AWZIs) worden toegepast zijn voor een aantal van deze stoffen en/of afbraakproducten (transformatie producten, TPs) onvoldoende, met als gevolg dat zij bij lozing terecht kunnen komen in het oppervlakte water. Het kunnen bepalen van drugs en hun metabolieten in de watercyclus draagt bij aan het begrijpen van de mogelijke impact van deze stoffen op het ecosysteem. Daarnaast geeft data van deze stoffen in het rioolwater informatie over het gebruik van drugs en patronen (trends) hierin. Om deze stoffen op het gewenste zeer lage niveau in rioolwater te kunnen meten zijn echter geavanceerde analytische methodieken nodig.

In dit proefschrift is onderzoek gedaan naar het potentieel van moderne analytische technieken, gebaseerd op de koppeling van vloeistofchromatografie aan tandem massaspectrometrie (LC-MS/MS) met triple quadrupool (QqQ), hybrid quadrupool time-of-flight (QTOF) en lineaire ionentrap Orbitrap (LTQ-Orbitrap) massa analysatoren voor de bepaling van drugs en hun metabolieten/TPs in verschillende type water monsters.

Het proefschrift bestaat uit vijf onderdelen en is het resultaat van negen wetenschappelijke artikelen en één hoofdstuk van een boek, geschreven op uitnodiging.

In het eerste deel werd het potentieel van UHPLC-MS/MS met triple quadrupool onderzocht voor de kwantitatieve bepaling van frequent gebruikte drugs en metabolieten in water. Twee multi-residu methoden, die gebaseerd zijn op een voorbehandelingstap met off-line vastefase-extractie (SPE) gevolgd door de instrumentele analyse met UHPLC-MS/MS, zijn hiervoor ontwikkeld. In beide methoden konden verschillende klassen van drugs, met uiteenlopende fysisch-chemische eigenschappen gekwantificeerd en geïdentificeerd worden in één enkele analyse-run. In de eerste methode werden 11 basische/zure analieten behorend tot drie

verschillende klassen drugs (amfetamine, cocaïne en cannabis) geanalyseerd. Deze drugs waren geselecteerd op grond van hun veelvudig gebruik in het Middellandse zee gebied. De methode is later geactualiseerd met toepassing van een nieuw ontwikkeld UHPLC-MS/MS instrument en heeft geresulteerd in de methode gepresenteerd in het tweede werk. Onder andere de de scoop werd uitgebreid, door inclusief verscheidene opiaten en ketamine te meten. De selectie van deze drugs was gemaakt op basis van gegevens verkregen van een uitgebreid Europees meetprogramma, waar, bijvoorbeeld, ketamine incidenteel werd gedetecteerd in rioolwater monsters. Lopende het onderzoek werd een gedetailleerde studie uitgevoerd om matrix-effecten te kunnen minimaliseren, die een negatieve invloed hebben op het resultaat van zowel de chromatografische scheiding als de gevoeligheid van de detectie. Beide methoden werden volledig gevalideerd en toegepast voor de analyse van stedelijk afvalwater van AWZIs gelegen in de provincie van Castellón, Spanje. Sommige resultaten maakten deel uit van een uitgebreid Europees onderzoek, waar ons laboratorium ook aan deelnam, ondersteund door het Europees Waarnemingscentrum voor Drugs en Drugsverslaving (EMCDDA). In het algemeen werden cocaïne en cannabis het meest frequent gedetecteerd en duidden de resultaten op een toename van het cocaïne gebruik gedurende de weekenden. Verder werd er een toename van de meeste drugs waargenomen gedurende een belangrijk muziek festival in Benicassim. Naast de analyse van drugs in rioolwater kon de mate van verwijdering van iedere analiet door de AWZIs (het zuiveringsrendement) geschat worden. Dit werd gedaan met behulp van de gemeten concentraties van drugs in ongezuiverd (influent) en gezuiverd (effluent) rioolwater. Voor de gewoonlijk voorkomende lage gehaltes was de verwijdering van deze stoffen uit het rioolwater voldoende. Echter, gedurende de week van het pop/rock festival, ging de forse toename in het gehalte aan drugs in het rioolwater gepaard met een afname van de verwijderingscapaciteit van AWZI.

Het tweede deel richt zich op het potentieel van LC gekoppeld aan Hoge-Resolutie Massaspectrometrie (HRMS) om de aanwezigheid van drugs in rioolwater te onderzoeken. De veelzijdigheid van LC, in combinatie met het hoog massa onderscheidendvermogen (resolutie) en zeer nauwkeurige massa bepaling van HRMS,

en de mogelijkheid om MS/MS of pseudo MS/MS experimenten uit te voeren, maakte het mogelijk om drugs en hun metabolieten gelijktijdig te detecteren en te identificeren met de gewenste selectiviteit en meetgevoeligheid. Allereerst werd het potentieel van UHPLC-QTOF MS onderzocht voor een snelle kwalitatieve analyse (screening) van 76 drugs, voorgeschreven geneesmiddelen met een potentieel risico op misbruik en enkele van hun metabolieten in rioolwatermonsters uit Spanje. Analyses zijn in MS^E acquisitie mode uitgevoerd waarbij twee spectra gelijktijdig worden verworven door middel van twee functies met verschillende botsingsenergieën. Dit maakte het mogelijk om data van zowel geprotoneerde moleculen (lage botsingsenergie) als fragment ionen (hoge botsingsenergie) te verkrijgen in één enkele analyse. Met deze screeningsmethode konden tien stoffen in influent- en effluent-rioolwatermonsters worden geïdentificeerd, waarvan (voorlopig) vijf stoffen (codeïne, cotinine (metaboliet van nicotine), ketamine, oxazepam en temazepam) zelfs zonder de beschikbaarheid van referentie standaarden. In een tweede studie werd de kwalitatieve en kwantitatieve prestaties van een LTQ Orbitrap massa analysator voor 24 drugs en relevante metabolieten in rioolwater bestudeerd gebruikmakend van de full-scan accurate massa data en gelijktijdige MS^n metingen. Bruikbare informatie van productionen werd gegenereerd door de LTQ. Validatie toonde aan dat de Orbitrap MS voldoende kwantitatieve capaciteiten heeft voor de bepaling van drugs op sub-ppb niveau. De methodologie werd toegepast op rioolwatermonsters van Nederland. Verscheidene drugs zoals MDMA, benzoylecgonine (BE), codeïne en benzodiazepines konden worden geïdentificeerd en gekwantificeerd. Met de MS data verkregen met Orbitrap kon bovendien retrospectieve onderzoek naar ketamine metabolieten in de monsters verricht worden.

Het derde deel betreft accurate massa spectra, verkregen met LC-QTOF MS in MS/MS en MS^E mode, waarmee enerzijds de fragmentatie van verscheidene drugs en hun metabolieten onderzocht kon worden en anderzijds de structuren van TPs van cocaïne en zijn voornaamste metaboliet BE, gevormd door degradatie in water, konden worden opgehelderd. Allereerst werden de fragmentatie routes onderzocht van verschillende typen drugs (amfetamines, cocaïne, opiaten, cannabinoiden en ketamine) en gerelateerde substanties, inclusief metabolieten en gedeutereerde

analogen. Door toepassing van accurate massa data en fundamentele fragmentatie concepten en regels konden chemische structuren van fragmentionen zorgvuldig worden gepostuleerd. Eerder gerapporteerde structuren en fragmentatiepatronen, die gebruik maakten van nominale massa data konden worden bevestigd met behulp van de geproduceerde accurate massa data. Daarnaast gaven deze data nieuwe inzichten in de fragmentatie van amfetamine en van (aan amfetamine) verwante stimulerende middelen, de voornaamste metaboliet van cannabis en opiaten. Daarna is een onderzoek uitgevoerd naar de TPs van cocaïne en BE. Gedurende behandelingsprocessen van rioolwater of door de blootstelling aan natuurlijke elementen in het milieu (bijvoorbeeld zonlicht) worden mogelijk TPs geformeerd, hiermee moet rekening worden gehouden, omdat deze stoffen wellicht persistenter zijn en/of een vergelijkbare toxiciteit hebben in vergelijking met de oorspronkelijke analiet en dus een belangrijke bijdragen kunnen hebben aan de impact van drugs op het milieu. Cocaïne werd geselecteerd omdat deze veelvuldig gebruikt wordt en omdat gewoonlijk hoge concentraties cocaïne en BE worden gevonden in influent rioolwater. In dit onderzoek werden verscheidene degradatie experimenten uitgevoerd onder laboratorium gecontroleerde condities. De accurate massa data en beschikbare kennis van fragmentatie van cocaïne en BE ondersteunden de structuuropheldering van verschillende TPs. Verscheidene bekende metabolieten / TPs konden worden bevestigd en andere onbekende stoffen konden (voorlopig) worden geïdentificeerd, zelfs wanneer geen referentie standaarden beschikbaar waren. Zestien en tien TPs van respectievelijk cocaïne en BE werden aangetoond. Vier niet eerder gerapporteerde TPs werden gedetecteerd in effluent rioolwater en oppervlaktewatermonsters.

Het vierde deel van het proefschrift is gericht op de aanwezigheid van drugs in "real life" watermonsters. Hiervoor werden watermonsters afkomstig uit Spanje en Nederland geanalyseerd met toepassing van de kwantitatieve analytische meetmethoden die in het eerste en tweede deel van dit proefschrift werden ontwikkeld. Doelen hierbij waren (i) analyse van de geselecteerde drugs en metabolieten in de geselecteerde verschillende typen watermonsters, (ii) de mate van verwijdering van deze analieten door de AWZIs, en (iii) de potentiële impact van deze analieten op het waterig milieu. Veel aandacht is besteed aan de kwaliteitsaspecten van de verkregen gegevens. Zo

zijn voor de kwantificering en bevestiging Europese richtlijnen toegepast en werden kwaliteitscontrole monsters (mee)geanalyseerd met de analyse van iedere serie monsters. Voor het eerste deel van dit onderzoek werden drugs dagelijks bepaald in influent- en effluent-rioolwatermonsters van drie AWZIs gelegen in de provincie van Castellón, Spanje. Monsters werden in drie perioden van één week verzameld, inclusief één week dat samenviel met een internationaal muziek festival in Benicassim. Onder normale omstandigheden zijn de concentraties laag en was de mate van verwijdering bevredigend. Echter gedurende het festival werd een opmerkelijke toename in concentraties waargenomen, niet alleen in de influenten maar ook in de effluenten was er een toename in concentraties en daarmee een toename in de belasting van het waterig milieu. Vervolgens werd met de LTQ Orbitrap MS onderzoek gedaan naar het voorkomen van 24 drugs in rioolwatermonsters van vijf AWZIs gelegen in Nederland, inclusief het internationale vliegveld van Amsterdam (Schiphol). De dagelijkse verschillen in belasting van drugs van het rioolwater en de mate van verwijdering werden geschat voor iedere drug en individuele AWZI. Door deze resultaten werd een beter inzicht verkregen van de aanwezigheid van drugs in Nederlands rioolwater en werd aangetoond dat de concentraties van drugs in rioolwater van het vliegveld slechts een klein verschil vertoonde met de concentraties gevonden in andere AWZIs. Voor de geanalyseerde drugs was het zuiveringsrendement van de AWZIs voldoende, behalve voor benzodiazepinen en MDMA. In een ander onderzoek werd een uitgebreide screening van 34 drugs en hun relevante metabolieten uitgevoerd in monsters van het Nederlandse watercyclus. Dit onderzoek werd uitgevoerd in samenwerking met vier laboratoria. Stedelijk afvalwater (influent en effluent), oppervlaktewater van de Rijn en Maas en drinkwater geproduceerd met de inname van water uit Rijn en Maas werden geanalyseerd. De resultaten lieten zien dat een substantieel deel van de totale lading van drugs in de Rijn en Maas vanuit het buitenland Nederland inkomt. Voor sommige onderzochte analieten, bleken ladingen stroomafwaarts te zijn toegenomen, wat lijkt te duiden op een bijdrage van Nederlandse rioolwater effluenten. In drinkwater werd in slechts één monster alleen de stof BE gedetecteerd in een concentratie beneden de bepalingsgrens. Op basis van dit onderzoek kon het toxicologische risico voor het milieu en voor volksgezondheid verwaarloosbaar worden geacht. Echter, aanbevolen wordt toekomstig onderzoek te

doen aangaande de mogelijke lange termijn (chronische) effecten op levende organismen en mogelijke effecten van gecombineerde blootstelling aan een veelvoud van stoffen in lage concentraties.

Het vijfde deel behoort formeel niet tot de onderzoeksopdracht van dit proefschrift, maar is een belangrijke aanvulling op de resultaten ervan. Het betreft de oprichting van een Europees wijd netwerk en wordt hier als een korte samenvatting gepresenteerd. Het netwerk is opgericht om de aanwezigheid van drugs in rioolwater te meten om zodoende een schatting te kunnen maken van het gebruik van deze stoffen in de betrokken Europese steden. De PhD kandidaat is nauw betrokken bij dit netwerk en heeft een belangrijke rol binnen het netwerk met betrekking tot ondersteuning en advisering van het gebruik en ontwikkeling van complexe analytische meetmethoden. Geavanceerde analytische technieken, meestal gebaseerd op chromatografie gekoppeld aan massaspectrometrie, zijn cruciaal om accurate concentratie data, van drugs en gerelateerde stoffen die in het rioolwater terecht komen, te verkrijgen. De moderne analytisch chemie heeft hierbij een sleutelrol in het vergaren van meer kennis met betrekking tot inname en trends van drugs binnen een gemeenschap. De inspanningen hebben geleid tot drie wetenschappelijke artikelen en één rapport welke zijn toegevoegd in dit deel, als illustratieve voorbeelden van boven genoemde Europese samenwerking. Deze artikelen beklemtonen de verschillen in drugsgebruik in verscheidene Europese steden en geven een algemeen overzicht van de analytische meetmethoden meestal toegepast in dit vakgebied, alsmede de onzekerheden die gepaard gaan met de benadering van drugs epidemiologie door middel van rioolwater analyse.

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Chapter 5: Occurrence of illicit drugs in the aquatic environment

Chapter 6: European-wide collaboration

List of Abbreviations

6-MAM	6-MonoAcetylMorphine
ADC	Analog to Digital Converter
ADHD	Attention Deficit Hyperactivity Disorder
APCI	Atmospheric Pressure Chemical Ionization
API	Atmospheric Pressure Ionization
ATS	Amphetamine-Type Stimulants
BE	Benzoylecgonine
BEH	Ethylene Bridges Hybrid
CE	Cocaethylene
CID	Collision Induced Dissociation
C-trap	C-shaped ion trap
DBE	Double Bond Equivalent (see also eq.)
DDA	Data-Dependent-Acquisition
DOA	Drugs of Abuse
EAWAG	Swiss Federal Institute of Aquatic Science and Technology
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	Ecgonine Methyl Ester
EPA	Environmental Protection Agency
ESI	Electrospray ionization
EU	European Union
FIB	Festival Internacional de Benicàssim
FT	Fourier Transform
FWHM	Full Width at Half Maximum
GC	Gas Chromatography
GC-MS	Gas Chromatography coupled to Mass Spectrometry
GFC	Glass microfiber filters
HE	High collision Energy
HLB	Hydrophilic-Lipophilic-Balanced
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry

IDs	Illicit drugs
ILIS	Isotope labelled internal standards
IP	Identification Point
IT	Ion trap mass analyzer
IUPA	Research Institute for Pesticides and Water
IUPAC	International Union of Pure and Applied Chemistry
KWR	KWR Watercycle Research Institute
LC	Liquid Chromatography
LC-MS	Liquid Chromatography coupled to Mass Spectrometry
LC-MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LE	Low collision Energy
LOD	Limit of Detection
LOQ	Limit of Quantification
LR	Low Resolution
LTQ	Linear ion trap
LVI	Large Volume Injection
MC	Management Committee
MCX	Mixed-mode Cation-eXchange
MDA	3,4-MethyleneDioxyAmphetamine
MDEA	3,4-MethyleneDioxyEthylAmphetamine
MDMA	3,4-MethyleneDioxyMethAmphetamine (also known as ecstacy (XTC))
MeOH	Methanol
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	Mass-to-charge ratio
NIVA	Norwegian Institute for Water Research
NMR	Nuclear Magnetic Resonance
NP	Normal-phase
NPS	New Psychoactive Substances
nw-XIC	Narrow mass-window eXtracted Ion Chromatogram
OCPs	OrganoChlorine Pesticides
OH-THC	11-hydroxy-THC

PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	PolyChlorinated Biphenyls
PPCPs	Pharmaceuticals and personal-care products
ppb	Parts per billion
ppm	Parts per million
Q	Quadrupole mass analyzer
Q_1	First quadrupole mass analyzer
q	Collision cell
Q_2	Second quadrupole mass analyzer
QC	Quality control
Q/q ratio	Ratio between the quantification (Q) and confirmation (q) transition
QqQ	Triple quadrupole mass analyzer
QTOF	Quadrupole time-of-flight mass analyzer
R	Resolution
RE	Removal Efficiency (see also eq.)
RIVM	National Institute for Public Health and the Environment
RP	Reversed-phase
RSD	Relative Standard Deviation
RT	Retention Time
SBE	Sewage-based epidemiology
SCORE	Sewage Analysis CORe group Europe
SETAC	Society of Environmental Toxicology and Chemistry
SIM	Selected Ion Monitoring
S/N	Signal-to-Noise ratio
SPE	Solid Phase Extraction
SPM	Suspended Particular Matter
SRM	Selected Reaction Monitoring
STP	Sewage Treatment Plant, (see also WWTP)
TDC	Time to Digital Converter
THC	Δ^9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-Δ^9-THC
TIC	Total Ion Chromatogram

TOF	Time of Flight mass analyzer
TPs	Transformation products
UHPLC	Ultra High Performance Liquid Chromatography
UJI	University Jaume I
UNODC	United Nations Office on Drugs and Crime
UV	Ultraviolet
WG	Work Group
WHO	World Health Organization
WWTP	WasteWater Treatment Plant, (see also STP)
XIC	eXtracted Ion Chromatogram

List of Equations and Definitions of Terms

eq. Back-calculation:

$$\text{ID use} = \frac{\text{conc.} \times \text{corr.factor}_{\text{stability}} \times \text{flow rate} \times \text{corr. factor}_{\text{excretion}} \times 10^9}{\text{number of inhabitants}}$$

ID use expressed as mg/day per 1000 inhabitants

Concentration of ID in influent wastewater (ng/L)

Flow rate of the wastewater stream (L/day)

eq. Double bound equivalent (DBE) = $1 + C - \frac{1}{2}(H + F + Cl + Br + I) + \frac{1}{2}(N + P)$

eq. Mass accuracy (absolute mass error, mDa) = *accurate* mass – *exact* mass

Mass accuracy (relative mass error, ppm) = absolute error / *exact* mass $\times 10^6$

eq. Mass resolving power (FWHM) = $m/\Delta m$

m is *m*/z being measured

Δm is the width of the mass peak at half peak height

eq. Removal efficiency (%) = $(1 - (C_E/C_I))$ 100%

C_E is concentration in effluent wastewater from day (x + 1)

C_I is concentration in influent wastewater from day (x)

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t. Accurate mass: experimentally determined mass of an ion of a known charge, that can be used to determine the elemental composition to within limits defined by both the accuracy and precision of the measurement*

t. Deprotonated molecule: an ion formed by the removal of a proton from a molecule M to produce an anion represented as $[M - H]^-$.

t. Exact mass: calculated mass of an ion or molecule containing a single isotope of each atom, most frequently the lightest isotope of each element, calculated from the masses of these isotopes using an appropriate degree of accuracy*

- **t.** Fourier transform: a mathematical transformation of the time domain signal in a frequency domain signal which is converted to a mass spectrum based in the inverse relationship between frequency and *m*/z

- **t.** Isobaric compounds: compounds, which have the same nominal mass, but different elemental composition and thus different exact mass

- **t.** Isomeric compounds: compounds with different chemical structures, but the same elemental composition and thus the same exact mass

- **t.** Mass defect: the difference between the nominal mass and the monoisotopic mass of a molecule, atom or ion* ($M_{exact} - M_{nominal}$)

- **t.** Metabolites are the intermediates and products of biological transformation processes *i.e.* metabolism, all chemical processes in living organism producing energy and growth**

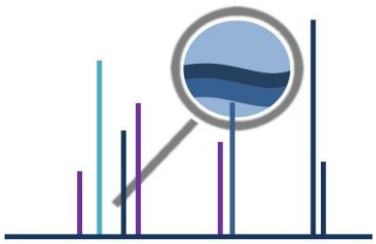
- **t.** Nominal mass: mass of an ion or molecule calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value and equivalent to the sum of the mass numbers of all constituent atoms*

- **t.** Protonated molecule: an ion formed by interaction of a molecule with a proton, i.e. where a molecule receives a proton represented by the symbolism $[M+ H]^+$

- **t.** Q/q ratio: ratio between the quantification (Q) and confirmation (q) transition

- **t.** Transformation products are the intermediates and products formed after physical, chemical and/or biological transformation processes**

* Standard definition of terms, IUPAC recommendations 2013 [Murray *et al.* 2013]
** In this thesis, we distinguish between metabolites formed in the human body and transformation products formed by physical, chemical and/or biological processes other than human metabolism



Objectives, methodology and working plan

(English – Español - Nederlands)

Objectives

The **main objective** of this doctoral thesis was to investigate the analytical capabilities of liquid chromatography coupled to mass spectrometry for the determination of illicit drugs and their metabolites/transformation products in waters. Different instrumental configurations have been investigated, including tandem MS, using triple quadrupole (QqQ), quadrupole-time of flight (QTOF) and linear ion trap Orbitrap (LTQ orbitrap) mass analysers.

In order to reach this general objective, several **specific objectives** have been established:

1. Development of an analytical methodology for the simultaneous quantification and reliable identification of illicit drugs (IDs) and their metabolites in waters (mainly sewage water) based on off-line solid phase extraction (SPE) followed by LC-MS/MS measurement with triple quadrupole and LTQ Orbitrap mass analyzers. Target analytes were selected on the basis of their high consumption and on previous data reported in the literature.
2. Optimization of the SPE conditions that allow the simultaneous and efficient pre-concentration of the selected analytes, without appreciable breakthrough.
3. Evaluation of matrix effects in quantitative LC-MS/MS methods for different types of waters. Correction of matrix effects, paying special attention to the use of isotope-labeled internal standards.
4. Development of a qualitative analytical methodology based on ultra-high-performance liquid chromatography (UHPLC) coupled to QTOF MS under MS^E mode, which allows rapid wide-scope screening for a large number of compounds in a single experiment.
5. Investigation of fragmentation pathways of different classes of IDs making use of UHPLC-QTOF MS. Elucidation of TPs of cocaine and its main metabolite benzoylecgonine, performing laboratory degradation experiments in water under controlled conditions. Analysis of wastewater and surface water samples in order to investigate the presence of the TPs previously identified in laboratory experiments.

6. Application of the developed analytical methodologies to influent and effluent urban wastewater from different geographic origin (Spain, the Netherlands and Germany). Realistic overview of the presence of IDs in water from the catchment areas studied. Estimation of the removal efficiency of the treatment processes applied by the WWTPs for the IDs under study. Evaluation of the potential environmental impact from data obtained in analysis of effluent wastewater and surface water.
7. Application of strict criteria for quality control and quality assurance of the compounds detected, making use of current European guidelines on quantification and confirmation. Analysis of quality control samples in every sequence of sample analysis. Participation in inter-laboratory exercises promoted and coordinated by the SCORE group.
8. Application of the developed analytical methodologies to influent urban wastewaters within a European wide-collaboration in order to back-calculate drug consumption and evaluate spatial differences and temporal changes in illicit drug use in Europe.

Methodology and working plan

The **methodology** applied for the development of **quantitative methods** was the following:

1. Selection of the target IDs and metabolites based on information of its consumption and that obtained from the scientific literature
2. Optimization of MS and MS/MS conditions by infusion of individual analytical standards in solvent.
   - Acquisition of MS spectra in full-scan establishing the most appropriate ionization mode and the cone voltage for the precursor ion.
   - Improvement of the formation of the protonated molecule (positive ionization mode) by using additives compatibles with the system (formic acid or ammonium acetate) when necessary.

- Isolation of the precursor ion and acquisition of product ion spectra. Optimization of collision energy. Selection of the most appropriate product ions taking into account the sensitivity (ion abundance) and selectivity (specifity of the transition), trying to avoid transitions derived from common losses such as the loss of water, carbon dioxide, formic acid, etc.
- Selection of, at least, two MS/MS transitions per compound to ensure the correct identification of the compounds detected in the samples. In the case of LTQ Orbitrap MS, the accurate mass of the precursor ion and at least one nominal mass product ion are used together with retention time.

3. Selection of the chromatographic conditions by injecting standard solutions. Selection of the mobile phase and gradient in order to obtain satisfactory chromatographic peaks and retention times.
4. Study of the SPE process efficiency for pre-concentration of the water samples, from comparative analysis of blank samples spiked before and after the SPE step.
5. Analysis of spiked SPE sample extracts of different types and origin, as well as of standards in solvent at the same concentration, in order to evaluate matrix effects on analyte ionization. Study of possible solutions for matrix effects correction that do not involves an increase of analysis time or sample manipulation, if feasible.
6. Validation of the optimized methods by evaluating linearity, accuracy and precision from recovery experiments at different concentration levels. Estimation of the limits of detection and limits of quantification of each compound.
7. Application of the developed analytical methodology to the analysis of different types of water samples (influent wastewater, effluent wastewater and surface water).
8. Confirmation of the identity of the compounds detected in the samples by evaluation of the ion intensity ratio for the selected transitions/product ions, together with retention time compliance in comparison with reference standards.
9. Discussion of the results obtained and establishment of the conclusions related to the presence of IDs and metabolites in the water samples analyzed.

The **methodology** used for **qualitative analysis** and **elucidation of TPs** in laboratory degradation experiments was the following:

1. Wide scope screening of IDs and metabolites
   a) Building of a comprehensive database. Initially, the name and elemental composition of target analytes was the only information included.
   b) Injection of the sample in UHPLC-QTOF MS operating in MS^E acquisition mode, which allows to simultaneously obtain full-spectrum accurate mass data at low and high collision energy.
   c) Data processing with specialized software *i.e.* ChromaLynx (within MassLynx v 4.1; Waters Corporation). This software facilitates a wide-scope screening by automatically extracting the exact mass of the protonated molecule from the spectrum at low energy, generating an extracted ion chromatogram (XIC). XICs are generated, after acquisition, of every compound listed in the customized database. The presence of a compound in the sample leads to the corresponding chromatographic peak.
   d) Calculation of the mass errors by comparison of the accurate mass with the theoretical exact mass.
   e) Identification of relevant fragment ions in the spectrum at high energy (if the investigated compound is really present in the sample, the retention time of the chromatographic peaks obtained after performing XICs at the exact masses of the fragments should be the same as for the protonated molecule and should show the same peak shape).
   f) Calculation of the mass error of the fragment ions.
   g) If commercial reference standards are available, then a comparison of their exact masses, fragment ions and retention times will be made for the unequivocal identification of the compound detected in the samples.
   h) If reference standards are not available, a tentative identification can be made on the basis of the information provided.
   i) Retrospective analysis of water samples, by including new drugs, TPs and fragment ions in the database. The latter for confirmative purposes.

2. Investigation of fragmentation pathways of IDs and related substances.
    a) Injection of standards solutions in UHPLC-QTOF MS operating in MS/MS and MS^E acquisition mode.
    b) Comparison of data obtained in MS/MS and MS^E mode.
    c) Calculation of the possible elemental composition and mass errors of fragment ions, taking into account the structure of the parent compound
    d) Comparison of data of IDs and related substances of the same class.
    e) Comparison with fragmentation pathways proposed in the literature, if available.
    f) Proposing structures of product ions using the accurate mass data provided by LC-QTOF MS, together with basic fragmentation concepts and rules.
3. Investigation of TPs
    a) Performing degradation experiments (hydrolysis, chlorination and photo-degradation (both ultraviolet irradiation and simulated sunlight)) under laboratory-controlled conditions.
    b) Injection of sample aliquots in UHPLC-QTOF MS operating in MS^E acquisition mode.
    c) Data processing with specialized software *i.e.* MetaboLynx (within MassLynx v 4.1; Waters Corporation). This software compares the chromatograms of the analyte sample and the control sample, and highlights differences in the presence of compounds, which could be attributed to transformation processes.
    d) Identification of relevant fragment ions in the spectrum at high energy
    e) Calculation of the mass error of the fragment ions.
    f) Structures of TPs are deduced by calculating the possible elemental composition from their measured accurate mass and by the application of suitable software (MassFragment) or by prediction, taking into account the structural differences with the parent illicit drug, and evaluation of the information by means of bibliographic search.
    g) If commercial reference standards are available, then a comparison of their exact masses, fragment ions and retention times will be made for the unequivocal identification of the compound detected in the samples.

h) If reference standards are not available, a tentative identification can be made on the basis of the information provided.

The **working plan** followed in the thesis was:

1. Selection of the IDs and metabolites to be investigated according to several criteria: most widely consumed drugs in the province of Castellón; drugs which were of major interest for local authorities; most relevant metabolites, as IDs are excreted with urine, either unchanged or as metabolite. In some cases the presence of a drug was studied through the presence of its metabolite(s) *i.e.* drug biomarkers. This information was obtained from a detailed literature search on IDs and metabolites detected in wastewater and surface water in studies reported by other authors; and on compounds with potential negative effects on the living organisms of the aquatic environment.
2. Bibliographic revision of the state-of-the-art on the current analysis methods for the determination of IDs and metabolites by LC-MS/MS, and practical aspects related to the selected techniques.
3. Development, optimization and validation of analytical methodologies based on UHPLC-MS/MS for the simultaneous determination of IDs and their metabolites in water. Detailed study of SPE process efficiency and on reduction of matrix effect in different types of water, evaluating the chromatographic performance and sensitivity. Correction of potential SPE losses and of matrix effects (enhancement/suppression of the signal).
4. Application of the developed quantitative analytical methodologies to wastewater samples (influent and effluent) collected from WWTPs located in the Castellon province, the Netherlands and Germany, and to surface water samples collected along the rivers Rhine and Meuse (the Netherlands).
5. Use of high resolution mass spectrometry (Orbitrap MS) for qualitative and quantitative analysis of IDs and relevant metabolites in urban wastewater, including retrospective analysis of data acquired (exact mass, full spectrum).

6. Application of the developed analytical methodology (point 5) to the analysis of wastewater samples (influent and effluent) collected from WWTPs located in the Netherlands. Evaluation of daily variances of drug loads and removal efficiencies for each drug and WWTP.
7. Development of analytical methodology based on UHPLC-QTOF MS under MS^E mode for wide-scope screening of IDs and their metabolites in urban wastewater.
8. Use of accurate mass full-spectrum data provided by high resolution mass spectrometry (QTOF MS) for the investigation of fragmentation pathways of various IDs and their metabolites.
9. Application of the previously developed strategy (point 7) for the identification and elucidation of TPs of cocaine and BE in water from experiments performed under laboratory controlled conditions and with knowledge acquired on common fragments (point 8).
10. Elaboration of the main conclusions derived from the research carried out in this thesis.

Objetivos

El **principal objetivo** de esta tesis doctoral es investigar las capacidades analíticas de la cromatografía líquida acoplada a espectrometría de masas para la determinación de drogas ilícitas y sus metabolitos/productos de transformación en aguas. Se han investigado diferentes configuraciones instrumentales, incluyendo MS en tándem, usando los analizadores de masas triple cuadrupolo (QqQ), cuadrupolo-tiempo de vuelo (QTOF) y trampa de iones lineal Orbitrap (LTQ orbitrap).

Para alcanzar este objetivo general, se han establecido varios **objetivos específicos**:

1. Desarrollo de metodología analítica para la cuantificación simultánea e identificación segura de drogas ilícitas (IDs) y sus metabolites en aguas (principalmente agua residual) basada en la extracción en fase sólida (SPE) *off-line* con posterior análisis por LC-MS/MS con analizadores de triple cuadrupolo y TLQ Orbitrap. Los analitos *target* fueron seleccionados en base a su alto consumo y en los datos previos reportados en la literatura.
2. Optimización de las condiciones de la etapa SPE que permite la preconcentración simultánea y eficiente de los analitos seleccionados, sin apreciable *breakthrough*.
3. Evaluación del efecto matriz en los métodos cuantitativos por LC-MS/MS para diferentes tipos de aguas. Corrección del efecto matriz, con atención especial al uso de patrones internos marcados isotópicamente.
4. Desarrollo de una metodología analítica cualitativa basada en cromatografía de ultra alta resolución (UHPLC) acoplada a QTOF MS en modo MS^E, el cual permite un rápido *screening* de un amplio espectro de compuestos en una sola experiencia.
5. Investigación de las vías de fragmentación de las diferentes clases de IDs haciendo uso de UHPLC-QTOF MS. Elucidación de TPs de la cocaína y su principal metabolito benzoilecgonina aplicando ensayos de degradación de laboratorio en agua bajo condiciones controladas. Análisis de muestras de agua residual y superficial para investigar la presencia de los TPs previamente identificados en los experimentos del laboratorio.

6. Aplicación de las metodologías analíticas desarrolladas a aguas residuales urbanas de entrada y salida de diferentes puntos geográficos (España, Países Bajos y Alemania). Visión realística de la presencia de IDs en el agua de las áreas de recogida de muestra estudiadas. Estimación de la eficacia de la eliminación de los IDs investigados en los procesos de tratamiento aplicados en las WWTPs. Evaluación del impacto medioambiental de los datos obtenidos en los análisis de agua residual de salida y de agua superficial.
7. Aplicación de un criterio estricto de control de calidad y de aseguramiento de la calidad de los compuestos detectados, en base a las directivas Europeas actuales en cuanto a cuantificación y confirmación. Análisis de muestras control de calidad en cada secuencia del análisis de muestras. Participación en ejercicios de interlaboratorio promovidos y coordinados por el grupo SCORE.
8. Aplicación de las metodologías analíticas desarrolladas en aguas residuales urbanas de entrada dentro de la colaboración europea para calcular el consumo de drogas y evaluar las diferencias espaciales y los cambios temporales en las drogas ilícitas usadas en Europa.

Metodología y plan de trabajo

La **metodología** aplicada para el desarrollo de los métodos cuantitativos fue la siguiente:

1. Selección de las IDs y metabolitos *target* en base a la información de su consumo y de la literatura científica.
2. Optimización de las condiciones MS y MS/MS mediante infusión del patrón analítico individual en solvente.
   - Adquisición de los espectros MS en *full-scan* estableciendo el modo de ionización más apropiado y el voltaje de cono para el ión precursor.
   - Aumento de la formación de la molécula protonada (modo de ionización positivo) mediante el uso de aditivos compatibles con el sistema (ácido fórmico o acetato amónico) cuando sea necesario.

- Aislamiento del ion precursor y adquisición del espectro de los iones producto. Optimización de la energía de colisión. Selección de los iones producto más adecuados atendiendo a la sensibilidad (abundancia del ión) y selectividad (especificidad de la transición), intentando evitar las transiciones derivadas de pérdidas comunes tales como pérdidas de agua, dióxido de carbono, ácido fórmico, etc.
- Selección de, al menos, dos transiciones MS/MS por analito para asegurar la correcta identificación de los compuestos detectados en las muestras. En el caso del LTQ Orbitrap MS, la masa exacta medida del ion precursor y al menos una masa nominal del ion producto son utilizadas junto con el tiempo de retención.

3. Selección de las condiciones cromatográficas mediante inyección de disoluciones patrón. Selección de la fase móvil y el gradiente para obtener picos cromatográficos y tiempos de retención satisfactorios.
4. Estudio de la eficacia del proceso SPE para la pre-concentración de las muestras de agua a partir de análisis comparativos de muestras blanco fortificadas antes y después de la etapa de SPE.
5. Análisis de extractos SPE fortificados de muestras de diferentes tipos y origen, así como patrones en solvente al mismo nivel de concentración para evaluar el efecto matriz en la ionización del analito. Estudio de posibles soluciones para la corrección del efecto matriz que no impliquen un aumento del tiempo de análisis o manipulación de la muestra, si es posible.
6. Validación de los métodos optimizados atendiendo a la linealidad, exactitud y precisión a partir de ensayos de recuperación a diferentes niveles de concentración. Estimación de los límites de detección y cuantificación de cada compuesto.
7. Aplicación de la metodología analítica desarrollada al análisis de diferentes tipos de muestras de agua (agua residual de entrada, de salida y agua superficial).
8. Confirmación de la identidad de los compuestos detectados en las muestras mediante la evaluación de la relación de intensidad de iones para las transiciones/iones producto seleccionados, junto con el cumplimiento de los tiempos de retención en comparación con los patrones de referencia.

9. Discusión de los resultados obtenidos y establecimiento de las conclusiones relacionadas con la presencia de IDs y metabolitos en las muestras de agua analizadas.

La **metodología** utilizada para **el análisis cualitativo** y **la elucidación de TPs** en los ensayos de degradación en el laboratorio fue la siguiente:

1. *Screening* de amplio espectro de IDs y metabolitos
   a) Creación de una extensa base de datos. Inicialmente, la única información incluida fue el nombre y la composición elemental de los analitos *target*.
   b) Inyección de la muestra en UHPLC-QTOF MS en modo de adquisición MS^E, el cual permite la obtención simultánea de los datos de masa exacta del espectro completo a baja y alta energía de colisión.
   c) Procesamiento de datos con software especializado *p.e.* ChromaLynx (de MassLynx v 4.1; Waters Corporation). Este software facilita un *screening* de amplio espectro extrayendo automáticamente la masa exacta de la molécula protonada del espectro de baja energía, generando un cromatograma de ion extraído (XIC). Los XICs son obtenidos, después de la adquisición, para cada compuesto incluido en la base de datos. La presencia de un compuesto en la muestra dirige al correspondiente pico cromatográfico.
   d) Cálculo del error de masa por comparación de la masa exacta medida con la masa exacta teórica.
   e) Identificación de los iones fragmento relevantes en el espectro de alta energía (si el compuesto investigado está presente en la muestra, el tiempo de retención de los picos cromatográficos obtenidos después de los XICs de los fragmentos de masa exacta debería ser el mismo que el de la molécula protonada y mostrar la misma forma de pico)
   f) Cálculo del error de masa de los iones fragmento.
   g) Si los patrones de referencia comerciales están disponibles, se realizará una comparación de sus masas exactas, iones fragmento y tiempo de retención

para la identificación inequívoca de los compuestos detectados en las muestras.

h) Si los patrones de referencia comerciales no están disponibles, puede hacerse una identificación tentativa en base a la información obtenida.

i) Análisis retrospectivo de las muestras de agua, incluyendo nuevas drogas, TPs, e iones fragmento de la base de datos. Estos últimos con fines de confirmación.

2. Investigación de vías de fragmentación de IDs y sustancias relacionadas.
   a) Inyección de disoluciones patrón en UHPLC-QTOF MS operando en modo MS/MS y MS^E.
   b) Comparación de los datos obtenidos en modo MS/MS y MS^E.
   c) Cálculo de la posible composición elemental y errores de masa de los iones fragmento, teniendo en cuenta la estructura del compuesto padre.
   d) Comparación de los datos de los IDs y sustancias relacionadas de la misma clase.
   e) Comparación con las vías de fragmentación propuestas en la literatura, si están disponibles.
   f) Propuesta de estructuras de iones producto utilizando los datos de masa exacta obtenidos mediante LC-QTOF MS, junto con los conceptos y reglas básicas de fragmentación.
3. Investigación de TPs.
   a) Realización de ensayos de degradación (hidrólisis, cloración y foto-degradación (ambos irradiación ultravioleta y simulación de la luz solar)) bajo condiciones controladas en el laboratorio.
   b) Inyección de alícuotas de muestra en UHPLC-QTOF MS operando en modo de adquisición MS^E.
   c) Procesamiento de datos con un software especializado *p.e.* MetaboLynx (de MassLynx v 4.1; Waters Corporation). Este software compara los cromatogramas de la muestra analito y de la muestra control, y resalta las diferencias en la presencia del compuesto, el cual podría ser atribuido a procesos de transformación.
   d) Identificación de los iones fragmento relevantes en el espectro de alta energía.

e) Cálculo del error de masa de los iones fragmento.
f) Deducción de las estructuras de los TPs mediante el cálculo de la posible composición elemental a partir de su masa exacta medida y aplicando el software adecuado (MassFragment) o mediante predicción, teniendo en cuenta las diferencias estructurales con la droga ilícita inicial, y evaluando la información a través de la búsqueda bibliográfica.
g) Si los patrones de referencia comerciales están disponibles, se realizará una comparación de sus masas exactas, iones fragmento y tiempo de retención para la identificación inequívoca de los compuestos detectados en las muestras.
h) Si los patrones de referencia comerciales no están disponibles, puede hacerse una identificación tentativa en base a la información obtenida.

El **plan de trabajo** seguido en la tesis fue:

1. Selección de las IDs y metabolitos para ser investigados de acuerdo a los siguientes criterios: amplio consumo en la provincia de Castellón; interés para las autoridades locales; metabolitos más relevantes, puesto que las IDs son excretadas en la orina como el producto inicial o como metabolito. En algunos casos la presencia de la droga fue estudiada a través de la presencia de sus metabolitos *p.e*. biomarcadores de drogas. Esta información fue obtenida a partir de una búsqueda detallada en la literatura de estudios reportados por otros autores en base a IDs y metabolitos en agua residual y superficial; y en compuestos con efectos potenciales negativos en los organismos vivos del medio acuático.
2. Revisión bibliográfica del estado del arte en los métodos de análisis actuales para la determinación de las IDs y metabolitos por LC-MS/MS, y los aspectos prácticos relacionados con las técnicas seleccionadas.
3. Desarrollo, optimización y validación de metodología analítica basada en UHPLC-MS/MS para la determinación simultánea de las IDs y sus metabolitos en agua. Estudio detallado de la eficacia del proceso SPE y reducción del efecto matriz en diferentes tipos de aguas, evaluando el proceso cromatográfico y la sensibilidad.

Corrección de las pérdidas SPE y del efecto matriz (ensalzamiento/supresión de la señal).

4. Aplicación de la metodología analítica cuantitativa desarrollada al análisis de muestras de agua residual (entrada y salida) recogida de las WWTPs situadas en la provincia de Castellón, Países Bajos y Alemania, y de muestras de agua superficial recogidas a lo largo de los ríos Rhine y Meuse (Países Bajos).
5. Uso de la espectrometría de masas de alta resolución (Orbitrap MS) para el análisis cualitativo y cuantitativo de IDs y metabolitos relevantes en agua residual urbana, incluyendo un análisis retrospectivo de los datos adquiridos (masa exacta, espectro *full scan*).
6. Aplicación de la metodología analítica desarrollada (punto 5) en el análisis de muestras de agua residual (entrada y salida) recogidas de las WWTPs situadas en los Países Bajos. Evaluación de las variaciones diarias de la carga de drogas y eficiencia de la WWTP en la eliminación de cada droga.
7. Desarrollo de metodología analítica basada en UHPLC-QTOF MS en modo MS^E para el *screening* de amplio espectro de IDs y sus metabolitos en agua residual urbana.
8. Uso de los datos del espectro *full scan* de masa exacta obtenido mediante espectrometría de masas de alta resolución (QTOF MS) para la investigación de las vías de fragmentación de varios IDs y sus metabolitos.
9. Aplicación de la estrategia previamente desarrollada (punto 7) para la identificación y elucidación de TPs de cocaína y BE en agua a partir de ensayos llevados a cabo en el laboratorio bajo condiciones controladas y con conocimiento adquirido en fragmentos comunes (punto 8).
10. Elaboración de las conclusiones principales derivadas de la investigación llevada a cabo en esta tesis.

Doelstellingen

De **hoofddoelstelling** van dit proefschrift was het analytische potentieel van vloeistofchromatografie gekoppeld aan massa spectrometrie te onderzoeken voor de bepaling van drugs en hun metabolieten/transformatie producten in water. Voor dit doel zijn verschillende instrumentele configuraties onderzocht, inclusief tandem MS, met triple quadrupool (QqQ), hybrid quadrupole time-of-flight (QTOF) en lineaire ionentrap Orbitrap (LTQ-Orbitrap) massa analysatoren.

Voor het bereiken van de hoofddoelstelling werden er verscheidene **specifieke doelstellingen** geformuleerd:

1. Het ontwikkelen van een analytische methodologie voor de gelijktijdige kwantificering en identificatie van drugs en hun metabolieten in water (hoofdzakelijk rioolwater), gebaseerd op off-line vastefase-extractie (SPE) gevolgd door LC-MS/MS metingen met QqQ en LTQ-Orbitrap massa analysatoren. De te analyseren stoffen waren geselecteerd op grond van hun hoge gebruik en van eerder gerapporteerde data in de literatuur.
2. Het optimaliseren van de SPE condities voor een gelijktijdige en efficiënte preconcentratie van de geselecteerde analieten.
3. De evaluatie van matrix-effecten (verhoging/onderdrukking van het signaal) die optreden in de kwantitatieve LC-MS/MS methoden voor verschillende typen water. Correctie van matrix-effecten, waarbij speciale aandacht wordt besteed aan het gebruik van isotoop gelabelde interne standaarden.
4. Het ontwikkelen van een kwalitatieve analytische methodologie gebaseerd op ultra-hoge-druk vloeistofchromatografie (UHPLC) gekoppeld aan QTOF MS in MS^E mode, voor een snelle uitgebreide screening van een groot aantal componenten in één enkel experiment.
5. Het onderzoeken van de fragmentatiepatronen van verscheidene klasse drugs gebruikmakend van UHPLC-QTOF MS. Het ophelderen van TPs van cocaïne en zijn voornaamste metaboliet BE d.m.v. het uitvoeren van degradatie experimenten onder laboratorium gecontroleerde condities. De analyse van rioolwater- en

oppervlaktewatermonsters om de aanwezigheid van de eerder geïdentificeerde TPs te onderzoeken.

6. Het toepassen van de ontwikkelde analytische methoden op zowel influent als effluent rioolwater van verschillende geografische oorsprong (Spanje, Nederland en Duitsland). Een realistisch overzicht maken van de aanwezigheid van drugs in afvalwater van de, in dit onderzoek, betrokken regio's. Een inschatting maken van de mate van verwijdering (het zuiveringsrendement) van drugs door verscheidene afvalwaterzuiveringsinstallaties (AWZIs). Een evaluatie maken van de mogelijke impact op het milieu op basis van data verkregen van de analyse van rioolwatereffluenten en oppervlaktewatermonsters.
7. Het toepassen van strikte eisen voor kwaliteitscontrole en kwaliteitsgarantie van de gedetecteerde stoffen, gebruikmakend van huidige Europese richtlijnen voor kwantificering en bevestiging. De analyse van kwaliteitscontrolemonsters, die met iedere groep monsters meegeanalyseerd worden. Deelname aan inter-laboratorium studies, zoals gepromoot en gecoördineerd door de SCORE groep.
8. Het toepassen van de ontwikkelde analytische methoden op influent stedelijk afvalwater binnen een Europese samenwerking om drug gebruik "terug" te berekenen en om ruimtelijke verschillen en tijdelijke veranderingen in het gebruik van drugs in Europa te evalueren.

Werkwijze en werkplan

Voor de ontwikkeling van **kwantitatieve methoden** werd de volgende werkwijze toegepast:

1. Selectie van de doelstoffen (drugs en metabolieten) gebaseerd op informatie over het gebruik en informatie verkregen van de wetenschappelijke literatuur.
2. Het optimaliseren van MS en MS/MS condities d.m.v. het uitvoeren van infusie experimenten van individuele analytische standaarden in oplosmiddel
   - Het verwerven van MS spectra in full-scan voor het selecteren van de geschikte ionisatie mode en "*cone voltage*" voor de "*precursor ion*".

- Het optimaliseren van de protonatie van het te bepalen molecuul (positieve ionisatie mode) door, indien nodig, gebruik te maken van additieven die toepasbaar zijn in het systeem (mierenzuur of ammonium acetaat).
- Het verwerven van gevoelige production spectra, door het isoleren van de precursorion en het optimaliseren van de botsingsenergie. De selectie van productionen, rekeninghoudend met de gevoeligheid (hoeveelheid geproduceerde ionen) en selectiviteit (specificiteit van de transitie), waarbij mogelijk transities afkomstig van gangbare verliezen, zoals het verlies van water, koolstofdioxide, mierenzuur, etc worden vermeden.
- Selectie van minstens twee MS/MS transities per doelstof (analiet) voor de correcte identificatie van de gedetecteerde stoffen in de monsters. Bij toepassing van de LTQ Orbitrap MS wordt voor de identificatie (i) de accurate massa van de precursorion, (ii) minstens één production in nominale massa en (iii) de retentie tijd gebruikt.

3. De selectie van geschikte chromatografische condities (mobiele fase samenstelling en gradientelutie) die resulteren in een adequate rententietijd en piekvorm van elk van de te bepalen analiet.
4. Een efficiëntie studie van het SPE proces voor de preconcentratie van de watermonsters, middels een vergelijkende analyse tussen blanco monsters verrijkt voor en na de SPE stap.
5. De bestudering van matrix-effecten door een vergelijkende analyse van verrijkte SPE extracten van monsters van verschillende typen water met die van standaardoplossingen met dezelfde concentraties. Studie naar de mogelijke oplossingen om matrix-effecten te corrigeren met behoud van de tijd (efficiëntie) van de totale analyse run.
6. De validatie van de geoptimaliseerde methoden door het bepalen van de lineariteit, juistheid en precisie middels het uitvoeren van recovery experimenten waarbij de analieten op verschillende concentraties zijn toegevoegd aan de matrix. De bepaling van de detectielimieten en bepalingslimieten van iedere analiet.
7. Het toepassen van de ontwikkelde analytische methoden voor de analyse van verschillende type watermonsters (influent rioolwater, effluent rioolwater en oppervlaktewater).

8. De bevestiging van de identiteit van de gedetecteerde stoffen in de monsters door de intensiteitsverhouding van geselecteerd transitie/product ionen te evalueren, samen met de overeenkomst van de retentietijd in vergelijking met referentiestandaarden.
9. Een discussie van de verkregen resultaten en het opstellen van de conclusies met betrekking tot de aanwezigheid van drugs en metabolieten in de geanalyseerde watermonsters.

De **werkwijze** voor **kwalitatieve analyse** en **opheldering van TPs** via laboratorium degradatie experimenten was als volgt:

1. Een uitgebreide screening van drugs en metabolieten
   a) Het opzetten van een omvangrijke database. In eerste instantie, wordt de naam en de elementaire samenstelling van de doelstoffen hierin opgenomen.
   b) Het analyseren van monsters in UHPLC-QTOF MS in de MS^E acquisitive mode voor het gelijktijdig verkrijgen van full-specutrm accurate massa data bij zowel lage als bij hoge botsingsenergie.
   c) Het verwerken van data met ChromaLynx software (MassLynx v 4.1; Waters Corporation). Deze software vergemakkelijkt een uitgebreide screening door automatisch de exacte massa van het geprotoneerde molecuul te extraheren uit het lage energie spectrum, daarmee genereerd het een "extracted ion chromatogram" (XIC). De XICs worden, na acquisitive, gegenereerd van iedere stof die in de lijst van de database staat. De aanwezigheid van een stof in het monster leidt tot de desbetreffende chromatografische piek.
   d) Het berekenen van de afwijkingen in exacte massa van een stof door de accurate massa te vergelijken met de theoretische exacte massa.
   e) De identificatie van een stof in een monster. Hierbij worden de mogelijke fragmentionen in het "hoge energie" spectrum onderzocht. De chromatografische pieken van de fragmenten (middels XICs van de exacte massa) moeten hierbij dezelfde de retentietijd en piekvorm hebben als die van het geprotoneerde molecuul.
   f) Berekening van de fouten in de massa van de fragmentionen.

g) Voor een éénduidige identificatie worden, bij beschikbaarheid van referentiestandaarden, de exacte massas, fragmentionen en retentietijden van de gedetecteerde stoffen in de monsters vergeleken met de waarden van die van referentiestoffen.
h) Indien referentiestandaarden niet beschikbaar zijn, wordt de stof voorlopig geïdentificeerd op basis van de verkregen informatie.
i) Het retrospectieve analyseren van watermonsters voor nieuwe drugs, metabolieten en TPs. Voor bevestigingsdoeleinden worden ook fragmentionen toegevoegd aan de database.

2. Het onderzoeken van fragmentatiepatronen van drugs en gerelateerde substanties door:
   a) De injectie van standardoplossingen in UHPLC-QTOF MS opererend in MS/MS en MS^E acquisitive mode.
   b) De vergelijking van data verkregen in MS/MS en MS^E mode.
   c) De berekening van de mogelijke elementaire samenstelling en massa fouten van fragmentionen, rekeninghoudend met de structuur van de onderzochte stof.
   d) De vergelijking van data van drugs en gerelateerde substanties van dezelfde klasse.
   e) De vergelijking van fragmentpatronen voorgesteld in de literatuur, mits deze beschikbaar zijn.
   f) Het voorstellen van structuren van productionen gebruikmakend van de accurate massa data verkegen met LC-QTOF MS, op basis van algemene fragmentatieconcepten en regels.
3. Het onderzoek naar de vorming van TPs door:
   a) Het uitvoeren van degradatie experimenten (hydrolyse, chlorering en foto-degradation (zowel ultraviolet bestralling als gesimuleerd zonlicht)) onder laboratorium gecontroleerde condities.
   b) De injectie van monsteraliquots in in UHPLC-QTOF MS opererend in MS^E acquisitive mode.
   c) Data verwerking met gespecialiseerde MetaboLynx (MassLynx v 4.1; Waters Corporation) software. Deze software vergelijkt chromatogrammen van een

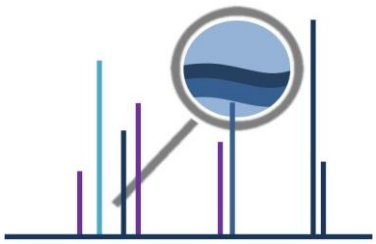
(met analiet verrijkt) monster met die van een controlemonster, en benadrukt verschillen in de aanwezigheid van stoffen. Wanneer een stof (chromatografische piek) aanwezig is in het verrijkte monster maar niet in de controle monster kan deze toegeschreven worden aan het transformatieprocessen.

d) De identificatie van relevante fragmentionen in het "hoge energie" spectrum.
e) De berekening van de afwijkingen in de massa van de fragmentionen.
f) Het herleiden van structuren van TPs door het berekenen van de mogelijke elementaire samenstelling van hun gemeten accurate massa en door de toepassing van geschikte software (MassFragment). Structuren kunnen ook worden voorspeld, rekening houdend met verschillen in structuur t.o.v. de onderzochte analiet. Daarnaast wordt de informatie geëvalueerd, d.m.v. een literatuur onderzoek.
g) Bij beschikbaarheid van referentiestandaarden zal er een vergelijking worden gemaakt van de exacte massas, fragmentionen en retentietijden voor eenduidige identificatie van de in de monsters gedetecteerde stoffen.
h) Bij afwezigheid van referentiestandaarden wordt de stof voorlopig geïndentificeerd op basis van de verkregen informatie.

Het **werkplan** van dit proefschrift was als volgt:

1. De criteria voor de selectie van de te onderzoeken drugs en metabolieten waren: (i) meest gebruikte drugs in de provincie Castellón; (ii) drugs die belangrijk waren voor lokale instanties; (iii) meest relevante metabolieten, aangezien drugs worden uitgescheden via urine, onveranderd of als metaboliet. In sommige gevallen wordt de aanwezigheid van drugs bestudeerd d.m.v. de aanwezighied van zijn metaboliet(en), d.w.z. drugbiomarkers. Deze informatie werd verkregen door een gedetailleerd literatuuronderzoek van drugs en metabolieten gedetecteerd in rioolwater en; (iv) stoffen die mogelijk negatieve effecten hebben op levende organismen van het waterig milieu.

2. Het maken van een literatuur up-date van de nieuwste ontwikkelingen van huidige analysemethoden voor de bepaling van drugs en metabolieten met LC-MS/MS, en aspecten gerelateerd tot de geselecteerde technieken.
3. De ontwikkeling, optimalisatie en validatie van analytische methoden gebaseerd op UHPLC-MS/MS voor de gelijktijdige bepaling van drugs en hun metabolieten in water. Gedetailleerde studie van de efficiëntie van het SPE proces en het minimaliseren van matrix-effecten in verschillende typen water, waarbij de impact van chromatografische prestatie en de gevoeligheid wordt geëvalueerd. Correctie van mogelijke verliezen veroorzaakt door SPE en/of matrix-effecten.
4. Het toepassen van de ontwikkelde analytische methoden op rioolwatermonsters (influent and effluent) verzameld van AWZIs gelegen in de provincie Castellón, Nederland en Duitsland, en oppervlaktewatermonsters verzameld langs de rivieren de Rijn en Maas (Nederland).
5. Gebruik van hoge-resolutie massaspectrometrie (Orbitrap MS) voor de kwalitatieve en kwantitatieve analyse van drugs en relevante metabolieten in rioolwater, inclusief retrospectieve analyse van de verkregen data (exacte massa, full spectrum).
6. De toepassing van de ontwikkelde analytische methode (punt 5) voor de analyse van rioolwatermonsters (influent and effluent) verzameld van Nederlandse AWZIs. De evaluatie van de dagelijkse verschillen in belasting door drugs en de mate van verwijdering voor iedere drug door de betreffende AWZI.
7. De ontwikkeling van een analytische methode gebaseerd op UHPLC-QTOF MS in MS^E mode voor een uitgebreide screening van drugs en hun metabolieten in rioolwater.
8. Gebruik van accurate massa full-spectrum data verkregen met hoge-resolutie massaspectrometrie (QTOF MS) voor het onderzoek naar fragmentatiepatronen van verscheidene drugs en hun metabolieten.
9. De toepassing van de eerder ontwikkelde strategie (punt 7) voor de identificatie en opheldering van TPs van cocaïne en BE in water middels experimenten uitgevoerd onder laboratorium gecontroleerde condities en met kennis verkregen van gemeenschappelijke fragmentionen (punt 8).
10. Het uitwerken van de voornaamste conclusies van het onderzoek uitgevoerd in dit proefschrift.



CHAPTER 1

General introduction

1.1 Illicit drugs

The origin of drug use and abuse relies on the fact that humans are inquisitive creatures. Drug use is as old as mankind itself and has become an integral part of human life, whether they are used for medicine, pleasure, religion, or just out of curiosity. Illicit drugs (IDs) or drugs of abuse (DOA) are a special group of widely consumed drugs. According to the United Nations Office on Drugs and Crime (UNODC), between 167 and 315 million people worldwide aged 15–64 were estimated to have used an illicit substance in 2010. This corresponds to between 3.6 and 6.9 per cent of the adult population [UNODC 2013].

Illicit drugs are not necessarily illegal, but it means that the drug is taken outside its regulated or medically prescribed use in an irresponsible or harmful way and might therefore cause severe harm to the user, others, or the society. Drug abuse has become a global problem with severe consequences, not only for the health and welfare of people, but also represents a clear threat to the stability and security of entire regions and to economic and social development. Accordingly, the term ID does not solely refer to consumption, but describes drugs which production, trafficking and use are under international control.

The IDs studied in this doctoral thesis are mostly illegal psychoactive drugs and can be classified as amphetamines, cocaine, opioids and cannabis. Psychoactive drugs affect

the central nervous system and stimulate or dull senses and promote a feeling of euphoria. In continue, brief general information on origin, pharmacokinetics and consumption of each group is described. It is noteworthy that the data available from human pharmacokinetic studies regarding the excretion profiles is limited for many IDs and the available clinical studies are generally incomplete and based on a small number of subjects. Furthermore, one should mainly take the urinary metabolism of a substance into account and percentages may differ depending on the route of administration, the habits of consumption, the amount of dose, and individual metabolism. Therefore, the percentage of excretion in urine should be taken and treated as indicative. More detailed information on IDs can be found in the literature [Wills 2005; Karch 2007; Castiglioni *et al.* 2008; EMCDDA 2012; UNODC 2013].

Amphetamines refer to a group of low-molecular weight basic stimulants whose principal members include ***amphetamine*** and ***methamphetamine***. However amphetamine has been the subject of intensive chemical manipulation. Quite small changes in chemical structure can result in derivates with significantly different properties. As a result, there is a large number of amphetamine-type stimulants (ATS) available, including compounds such as ***3,4-methylenedioxymethamphetamine*** *(*MDMA, commonly known as ecstasy), ***ephedrine*** and ***methcathinone***.

Amphetamines are sympathomimetic agents that release monoamines from nerve endings in the brain via the neurotransmitters noradrenaline, dopamine and serotonin. They have been used in the treatment of obesity, narcolepsy and attention deficit hyperactivity disorder (ADHD) of children, but now they are mainly used because of their stimulant activity. As an abusive drug in Europe, the north of Europe is suggested to have the highest prevalence of amphetamines use in the general population. Amphetamine is more commonly available in Europe than methamphetamine, but the latter has particularly appeared on the drug markets in the north and east of Europe, where it seems to have partially replaced amphetamine in the last few years [EMCDDA 2012]. Amphetamines are mainly excreted in the urine in unaltered forms (**Fig. 1.1**).

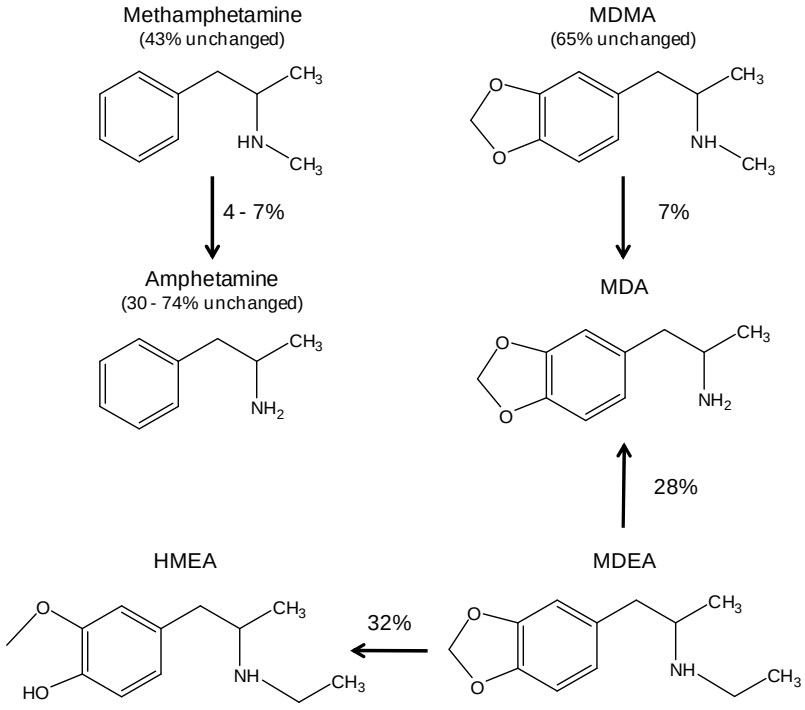


Figure 1.1. The metabolic correlations between amphetamines, with percentages of excretion in urine [Castiglioni *et al.* 2008]

Cocaine is a naturally occurring alkaloid, extracted from the leaves of the coca plant (*Erythroxylum coca Lam.*). Cocaine is cultivated widely on the Andean ridge in South America. Refined cocaine, in the form of hydrochloride salt or as free base (sometimes known as "crack") is the most common form of the drug used. Cocaine can be self-administered in many ways, including snorting, smoking and by injection. Levels of cocaine use in 2011 (2.7 %) reported by Spain, are above the European average (1.2 %), and prevalence data show that cocaine is the most commonly used illicit stimulant drug. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) also report that Spain, together with the Netherlands, Portugal and Belgium appear to be the main points of entry to Europe for cocaine [EMCDDA 2012].

Cocaine is one of the most potent stimulants. It has powerful central nervous system effects, similar to those of amphetamines. In the human body, cocaine is rapidly

metabolized by liver carboxylesterases to benzoylecgonine (BE, primary metabolite) and ecgonine methyl ester (EME). It is partially excreted as the unchanged drug and can be metabolized forming cocaethylene (CE) when used in combination with alcohol (ethanol). A scheme of its metabolism is shown in **Fig. 1.2**.

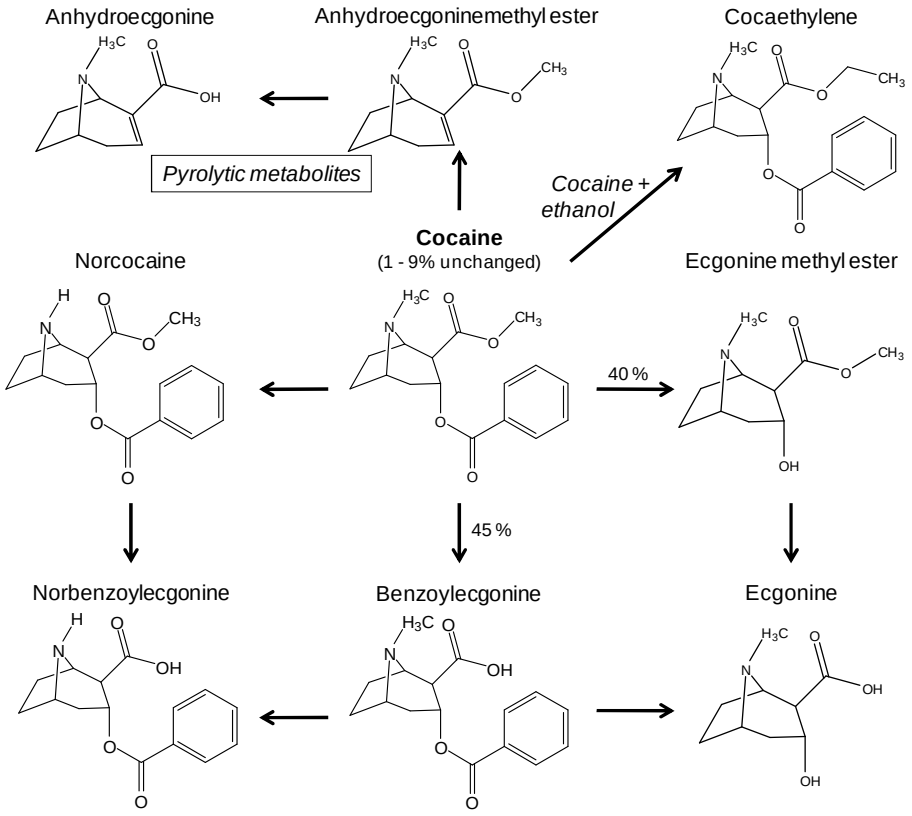


Figure 1.2. The metabolic pathway of cocaine, with percentages of excretion in urine as parent compound and metabolites [Castiglioni *et al.* 2008]

Opioids refer to a group of natural, semi-synthetic, or synthetic alkaloid drugs derived from the latex of the opium poppy (*Papaver somniferum*). Opiates are natural compounds and are among the oldest drugs in existence and were the first to be seen as a major public health treat. They include ***morphine*** and ***codeine***. The synthetic or

semi-synthetic opioids, such as ***methadone*** and ***fentanyl***, are similar to the opiates in their chemical structure and biological effects.

Opioids, such as morphine, codeine and ***oxycodone*** are commonly prescribed because of their effective analgesic and pain-relieving properties. However, non-medical use of these drugs is often reported. ***Heroin*** (produced by an acetylation of morphine) is the most widely abused opioid (about 75 % of the users), because of its potency, availability, solubility in water, and high biological lipophilicity, which permits rapid brain access. Recent national estimates vary between less than one and seven cases per 1000 population aged 15–64. In Spain, however, it does not seem to be a primary drug. Reports of heroin being replaced by other drugs, including synthetic opioids, such as fentanyl, but also the injection of stimulant drugs, including amphetamines is an increasing concern in health risk. Nevertheless, heroin is still responsible for the greatest share of morbidity and mortality related to drug use in the European Union (EU) [EMCDDA 2012].

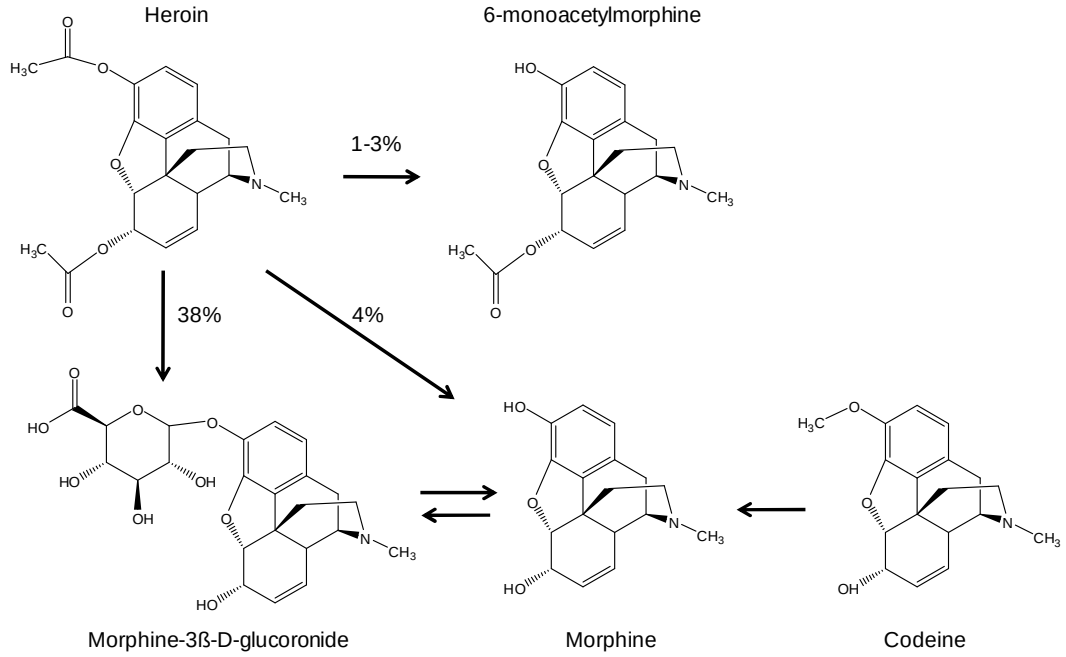


Figure 1.3. The metabolic pathway of heroin, morphine and codeine in man, with percentages of excretion in urine [modified from Castiglioni *et al.* 2008)]

In the human body, heroin is rapidly deacetylated to 6-monoacetylmorphine (6-MAM) and morphine in the liver (**Fig. 1.3**). Morphine is a metabolic residue common to heroin, codeine, and morphine itself, whereas 6-MAM (1 - 3% of a dose) is a specific metabolite of heroin [Baselt 2004; Castiglioni *et al.* 2008].

Cannabis is a natural product and is produced in virtually every country of the world in the form of cannabis herb (*Cannabis sativa L.*), making it the most widely produced and consumed illicit drug around the globe. Herbal cannabis (***marijuana***), the dried flower buds of the female cannabis plant, is mainly produced for domestic or regional markets, while the more processed and compressed resin of the cannabis plant is confined to far fewer countries. Afghanistan and Morocco are identified as the largest producers of cannabis resin (also known as ***hashish***), which enters Europe primarily through the Iberian Peninsula [EMCDDA 2012; UNODC 2013].

Cannabis are non-polar compounds with low solubility in water and therefore usually self-administered by smoking. The potency of cannabis products is determined by their content of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive constituent. Cannabis potency varies widely between and within countries, between different cannabis products and between genetic varieties. In 2010, the reported mean THC content of cannabis resin ranged from 1 % to 12 % and the mean potency of herbal cannabis ranged from 1 % to 16.5 % [EMCDDA 2012]. Spain is considered to be one of the countries with highest prevalence of Europe with estimates at about 20% of young adult consumers (mainly cannabis resin) aged 15 - 35 years [EMCDDA 2012].

Cannabis has both psychoactive and physiological effects on the human body. Effects include euphoria and relaxation but also a decrease in short-term memory and feelings of paranoia. Apart from recreational use, THC also has some therapeutic benefit as an analgesic and is prescribed (as dronabinol) in some countries for the treatment of nausea in cancer chemotherapy, and to people with chronic pain. THC is highly lipophilic and widely distributed in the human body. It is metabolized in the liver to the active metabolite 11-hydroxy-THC (OH-THC), which is further oxidized producing the inactive metabolite 11-nor-9-carboxy-Δ^9-THC (THC-COOH) (**Fig. 1.4**).

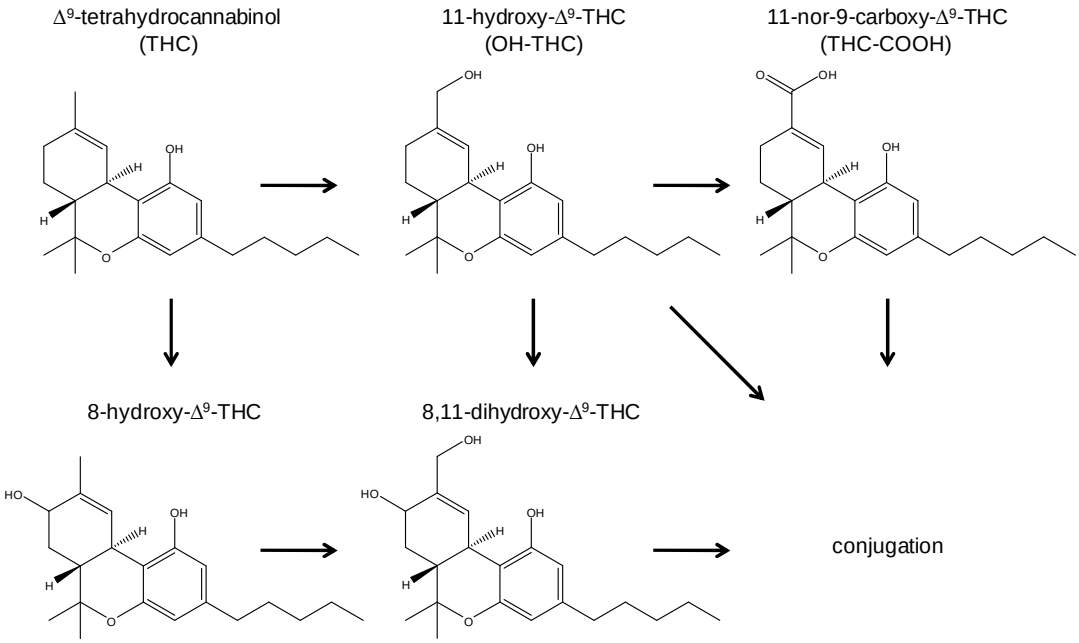


Figure 1.4. The metabolic pathway of Δ9-tetrahydrocannabinol [Castiglioni *et al.* 2008]

1.2 Illicit drugs in the aquatic environment: sewage-based epidemiology and environmental implications

Illicit drugs may enter the sewage system, unaltered or as a metabolite, after consumption and excretion. Recent technological advances in analytical chemistry have allowed developing sensitive and accurate methodologies based on chromatographic and mass spectrometric techniques to detect these substances in the aquatic environment at trace levels (ng/L). The determination of IDs and their metabolites in the water cycle can provide information on drug use and trends, but data from analysis can also contribute to the understanding of the potential impact of these compounds on the aquatic ecosystem. In general the IDs that have been determined in water include those with high worldwide levels of use.

1.2.1 Sewage-based epidemiology

Illicit drug use is stigmatized and often a hidden activity, as it is generally considered as socially undesirable behavior. Nonetheless, timely information about the trends and extent of ID consumption in the general population is important. Policymakers need accurate information so that they can make evidence-based decisions in order to reduce the high economical and social costs related with the use of these substances e.g. the prevention of drug use, the treatment and care of drug dependence, and the suppression of organized crime [EMCDDA 2012; UNODC 2013].

The prevalence of drug use is traditionally estimated by subjective methods such as general population surveys, interviews, monitoring drug-related criminality and hospital records [Wiessing *et al.* 2008]. Despite the considerable improvements using today´s communication facilities and the use of complementing methods such as targeted studies and statistical modeling, these survey methods mainly rely on the willingness of users to self-report and to participate in monitoring actions. The social taboo related to drug abuse, provokes that users do not want to participate in such surveys, which makes these methods vulnerable and often inaccurate. In addition, a common problem of all current methods is that they are time-consuming, expensive and complex. Therefore, the UNODC and EMCDDA encourage the development of new

complementary approaches in order to obtain objective, low-cost, fast, reliable and comparable data.

An innovative and promising approach to estimate, objectively, consumption of IDs in large communities is through the analysis of sewage water. "Sewage profiling for community-wide drug use and lifestyle assessment" also known as "sewage-based epidemiology" allows monitoring drug consumption in real-time, enabling quick identification of changing trends, which is essential to the identification of problems [EMCDDA 2008; van Nuijs 2012a].

The sewage-based epidemiology (SBE) approach is based on the principle that IDs consumed by individuals are excreted with urine, either unchanged or as metabolite, into urban sewer networks. Concentrations of drug biomarkers measured in influent wastewater collected at the inlet of a wastewater treatment plant (WWTP) reflect the amount of drugs collectively excreted by consumers and can be used to estimate drug abuse of that particular WWTP catchment area *i.e.* community [van Nuijs 2012a]. The concept of sewage epidemiology was first proposed by Daughton [Daughton 2001], but put into practice in 2005 by Zuccato and his co-workers. They reported the presence of cocaine and its major metabolite, benzoylecgonine, in influent sewage water of some Italian cities and estimated the levels of consumption of cocaine in those localities [Zuccato *et al.* 2005]. Obviously, consumption levels cannot be estimated by solely measuring ID concentrations in influent wastewaters. Several crucial steps are involved, starting with the collection of a representative sample. Different sampling modes, such as flow-, time- and volume-proportional modes, can be applied with the objective to obtain a 24-hour composite sample that is representative for a complete day [Ort *et al.* 2010]. Concentrations measured in the sample are to be multiplied with daily wastewater volumes, to calculate the total drug loads entering the wastewater system (expressed in g/day). The drug loads are then normalized by the number of people that are contributing to the catchment area of the WWTP to allow comparisons between the different localities *i.e.* back calculation of the amount of a drug used in a community. The transformation of the normalized loads into an amount of used illicit drug has then to be corrected with knowledge of the pharmacokinetic

data (excretion rates) of the substance and purity of drugs. The last stage of the approach allows estimation on the number of doses used in a certain population. An overview of the SBE approach is given in **Figure 1.5**.

SBE is a rapidly developing scientific discipline with potential for monitoring spatial and temporal differences in drug use. However the methodology is still at its infancy and in full development. Challenges arise from shortcomings [Baker and Kasprzyk-Hordern 2011; Baker *et al.* 2012] and uncertainties associated with sampling of sewage, behavior of the selected biomarkers in the sewer, reliability of inter-laboratory analytical measurements, different back-calculation methods, and different approaches to estimate the size of the population being tested [Castiglioni *et al.* 2013]. Ethical issues of wastewater analysis have also come under scrutiny [Hall *et al.* 2012]. Accordingly, SBE requires more extensive research and validation; until then, the results of wastewater studies need to be interpreted cautiously. Yet, the ability to provide independent, anonymous, low-cost, reliable and almost real-time estimates of ID consumption in a targeted population makes it a useful complement to existing monitoring tools [EMCDDA 2012; van Nuijs 2012a].

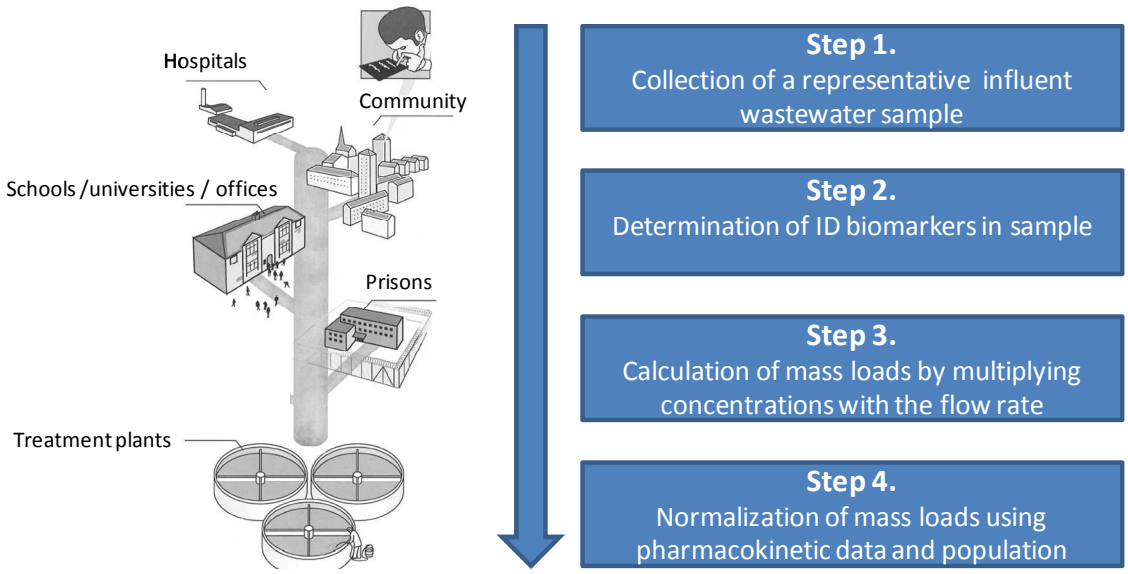


Figure 1.5. Sewage-based epidemiology

The IDs commonly studied are mostly illegal psychoactive drugs, although some of these drugs are used as therapeutic drugs, such as amphetamine, morphine and codeine, but they are also well known IDs, and have frequently been treated as such. As for SBE, pharmacokinetic data of IDs becomes especially important for proper drug biomarker selection and correct back-calculation of drug use. The compounds targeted for analysis of IDs in wastewater, *i.e.* sewage drug biomarkers, are generally the main urinary excretion products, either the unchanged parent drugs, or their most abundant metabolites.

Amphetamines are mainly excreted in the urine in unaltered forms. Thus, the parent compounds have generally been chosen as the analytical target. Oppositely, only 1–9% of a cocaine dose is excreted in urine as unchanged, while approx. 45% and 40% is excreted as BE and EME, respectively [Baselt 2004]. Moreover, BE is more stable than cocaine and EME in sewage [Castiglioni *et al.* 2013]. Consequently, BE is the biomarker commonly used for estimating cocaine use by wastewater analysis. According to Castiglioni *et al.* 2008, heroin is rapidly deacetylated to 6-MAM and to morphine. Analysis in wastewater is, therefore, focused on morphine and on this specific heroin metabolite 6-MAM. Morphine is partly excreted as glucuronide conjugates [Baselt 2004], which are, however, readily hydrolyzed back to morphine by beta-glucuronidases of fecal bacteria, as shown for glucuronide conjugates of estrogens in untreated wastewater [D'Ascenzo *et al.* 2003] and during wastewater treatment [Ternes *et al.* 1999]. THC-COOH, the main metabolite of cannabis, is excreted in substantial amounts in the urine mostly conjugated with glucuronic acid, but as with morphine, it is readily hydrolyzed back to THC-COOH by beta-glucuronidases of fecal bacteria in untreated wastewater and during wastewater treatment. Therefore THC-COOH is generally chosen as the analytical target for wastewater analysis [Castiglioni *et al.* 2008].

1.2.2 Environmental implications

The quality of water has an essential impact on human health and environment. Although it has improved in the last decade, classic contaminants like heavy metals, organochlorine pesticides (OCPs), polycyclic aromatic hydrocarbons (PAHs),

polychlorinated biphenyls (PCBs) or dioxins are still detected in environmental waters [de Nijs 2008]. In addition to these classic contaminants, the research on the presence of new emerging contaminants in the aquatic environment is considered important in order to assure the quality of water and has become a major concern for many authorities, water companies, public health and environmental organizations. The growth of new chemicals and industries, together with the continued rise of the world population, but also the increased sensitivity and selectivity of analytical methods has become the main reason for the detection of emerging contaminants in environmental waters.

The term "emerging contaminants" covers a wide group of compounds that are not yet under regulation. These include pharmaceuticals and personal-care products (PPCPs), hormones and other endocrine disrupting compounds, but also brominated flame retardants, perfluorinated compounds, artificial sweeteners, nanomaterials, disinfection products, biological transformation products, pesticide transformation products, and many other organic contaminants [Richardson and Ternes 2011]. Public health and environmental organizations, such as the Environmental Protection Agency (EPA) of the United States, the World Health Organization (WHO) and the European Commission periodically revise their priority lists of contaminants and incorporate those compounds most frequently detected in environmental waters.

In this context, IDs and their metabolites have been considered as a novel group of emerging environmental contaminants [Zuccato *et al.* 2008a; Richardson and Ternes 2011]. The amount of IDs consumed worldwide may be comparable in some cases with those of pharmaceuticals, as millions of individuals are current users of amphetamines, cocaine, opioids, cannabis and other drugs. Following their consumption and excretion, some IDs and/or their metabolites are continuously released into natural surface waters, due to their insufficient elimination in WWTP. Their occurrence in the environment is therefore increasingly recognized as an important issue.

Concentrations of IDs found in natural waters are generally low (sub ppb level), hence there is still a debate about toxicity for humans and wildlife (e.g. fish and plants).

Although no short-term effects are expected, long-term (chronic) effects on organisms and the possible effects of combined exposure to multiple compounds are not ruled out and are of concern [van der Aa *et al.* 2013]. Unlike pharmaceuticals, little attention has been devoted to the environmental fate and transport of IDs. Ecotoxicological information for IDs is scarce, especially with regard to low-level mixed-stressor exposure. Almost nothing is known regarding the potential for biological effects in aquatic systems, the bioconcentration or bioaccumulation of IDs in biota [Daughton 2011]. Therefore, a continuous survey of the levels in the aquatic environment is important to know their sources, fate, and persistence. However, not only the parent compounds and human metabolites must be taken into account, but also the possible formation of degradation/transformation products (TPs). These products may be formed due to the exposure to natural elements in the environment (e.g. sunlight, temperature) or during wastewater treatment processes. Compared to the classic pollutants (e.g. persistent organic pollutants, like OCPs, PCBs, dioxins, or PAHs, among others), the new contaminants are usually more polar and thus more soluble in water. They tend to stay in water and are consequently more difficult to remove or eliminate during conventional water treatment processes. For this reason, WWTPs frequently apply additional treatment based on advanced procedures, such as oxidation and ultraviolet (UV) irradiation, to remove these compounds. Despite the advantages of additional treatment, this may also result in the formation of disinfection by-products, as the complete degradation of the contaminant is not likely to occur in most cases. Some of these TPs may be more persistent or might even exhibit similar toxicity than their parent compounds [Farré *et al.* 2008; Fatta-Kassinos *et al.* 2011], and should therefore be investigated and included in monitoring studies in order to know the overall contribution of chemicals in the environment. The data generated will allow performing a well-founded scientific risk assessment in the future. Furthermore, the performance of WWTP needs to be continuously re-evaluated and optimized, based upon new research results.

Sophisticated analytical methodologies able to reveal the presence of IDs, metabolites and TPs in the aquatic environment, are required to face all these problems. The reliable identification of the compound detected is of outstanding relevance,

especially when dealing to a large number of compounds, in some cases with close similarities in their chemical structure. Also the accurate quantification is needed to have a better knowledge of the environmental risks.

1.3 Role of LC-MS in the determination of illicit drugs in water

The determination of IDs and metabolites in water samples requires the use of highly sensitive and selective analytical procedures capable of quantifying concentrations in the low ng/L to µg/L range. To achieve this goal, a sample clean-up and/or analyte concentration steps are normally necessary, followed by chromatographic separation of the analytes, detection, quantification and confirmation.

A crucial step is the application of proper detection technologies that should be specific and at the same time sensitive enough. Gas chromatography coupled to mass spectrometry (GC-MS) [Mari *et al.* 2009; González-Mariño *et al.* 2010] in general provides high levels of selectivity and sensitivity. However, derivatization of target compounds is often necessary for most of IDs in order to make analytes compatible with the GC requirements. As a consequence, sample pre-treatment is generally laborious. Liquid chromatography coupled to mass spectrometry (LC-MS) is a more versatile technique with less sample treatment and shorter run times that allows the determination of polar, low-volatility and/or thermo labile compounds, like most drugs are. Therefore, liquid chromatography coupled to mass spectrometry, or better with tandem mass spectrometry (LC-MS/MS), mainly using triple quadrupole (QqQ) analyzers, has become the technique of choice for the determination of IDs in biological [Pizzolato *et al.* 2007] and water samples [Castiglioni *et al.* 2011; Van Nuijs *et al.* 2011a]. New analytical approaches using LC coupled to high-resolution and high-accuracy mass spectrometry (HRMS) have recently been explored, showing strong potential for target and non-target screening. HRMS has proved to be an efficient tool for the identification/elucidation of IDs and metabolites at low concentrations in complex water samples. Although less explored than LC-MS/MS QqQ, HRMS also allows quantification of IDs with satisfactory performance.

Reliable data on IDs and metabolite concentrations in sewage water (influent and effluent), surface water and drinking water are the basis of the subsequent calculation of drug loads and consumption, the removal efficiencies in WWTPs, and to estimate the discharges into the aquatic environment. The most relevant aspects within the

analytical methodology that affect accurate quantification and reliable identification are briefly commented in the following sections.

1.3.1 Sample pretreatment

When dealing with the determination of IDs in water samples, the low analyte concentrations and the complexity of some sample matrices generally make a pre-concentration and/or a clean-up step necessary before instrumental analysis in order to achieve the required selectivity and sensitivity.

Off-line solid-phase extraction (SPE) is mostly applied for this purpose and it is considered one of the most versatile and reliable techniques. It allows the easy adjustment of the sample volumes (normally between 50 and 1000 mL) and the elution conditions to obtain the desired sensitivity and to maximize the recoveries of different compounds [Bagnati and Davoli 2011].

A filtration or centrifugation step to remove solid particles is generally recommended before loading the sample into the cartridge to prevent the SPE material from clogging. This is especially true when treating sewage water samples. Subsequently, SPE conditions (*i.e.* conditioning, loading, washing and eluting) need to be optimized, and a compromise of these experimental conditions must be found when different groups of analytes are measured. Depending on the SPE sorbent material and procedure, the pH of the sample may be adjusted to increase the affinity of the analytes with the sorbent and therefore improving the extraction efficiency.

The most important parameters affecting the extraction efficiency of a SPE procedure are the sample volume, the sample loading flow rate and the SPE sorbent material. Small sample volumes may compromise the method sensitivity, whereas large sample volumes may lead to sample break-through and also affect the method sensitivity negatively, due to the increased amount of matrix interferences. The sample loading flow rate *i.e.* the time of contact between the sample analytes and the sorbent surface, and thus the extraction efficiency of the SPE process may decrease with increasing flow rates.

Another relevant parameter is the elution step, as it is necessary to ensure that analytes are quantitatively recovered from the cartridge after having being retained. A variety of solvents can be used for the efficient elution of analytes. This step needs to be carefully tested to avoid unnecessary losses of analytes along the overall analytical process.

The ratio between sample volume loaded into the cartridge and elution volume, which is finally adjusted to a given final volume of the sample extract, gives the sample pre-concentration factor. This factor is normally adjusted to reach a compromise between sensitivity and matrix effects. Pre-concentration factor between 20 and 500 are normally used in the majority of methods reported in the literature.

SPE is a sorptive technique, where retention is due to interactions between the analyte and the cartridge material. Thus, the type of sorbent used is responsible for the efficiency of the extraction process. The numerous materials that can be used as SPE sorbents were reviewed by Fontanals *et al.* 2007. In general, the chemical structure and the morphology (*i.e.* specific surface area, distribution of pore diameter, particle size, etc) of the material are important, as they determine the type of interactions and the mechanical properties, respectively. Classical sorbents are silica-based (C_2, C_8, C_{18}), carbon-based or polymeric. However, the SPE cartridges typically used for the analysis of IDs in aquatic samples are based on the more recently developed mixed mode sorbent Oasis HLB and MCX. The more generic Oasis HLB cartridge is built of a balanced mixture of hydrophilic and lipophilic monomers. The specific surface area (~800 m^2/g) possesses a high number of points for interaction and its hydrophilic/lipophilic character makes it suitable for interaction with analytes of a wide range of polarity, ideally for multi-residue analysis of drugs with the different properties (acid, neutral, basic). On the other hand, Oasis MCX is a Mixed-mode Cation-eXchange polymeric sorbent built upon HLB copolymer that allows improved selectivity towards basic analytes. The compounds are retained by means of ionic interaction (sulfonic-acid-cation-exchange) and therefore an adjustment of the sample pH is commonly required to have the analytes protonated, able to be retained by the cartridge.

1.3.2 Liquid chromatography coupled to mass spectrometry

Liquid chromatography (LC) is a chromatographic separation technique, which makes use of the distinctive affinity of the components of the sample mixture, contained and transported by a liquid mobile phase, with the sorbent particles of a stationary phase. It is a repeated mass transfer process involving adsorption/desorption. Those components that are strongly retained by the stationary phase move slowly with the flow of the liquid mobile phase and *vice versa*. The differences in affinity and thus mobility, separates sample components into discrete bands that can be analyzed qualitatively and/or quantitatively [Christian 1994].

LC can operate in normal-phase (NP) as well as in reversed-phase (RP) mode. NP chromatography is often used for the analysis of relatively non-polar compounds, as the polarity of the stationary phase is higher than that of the mobile phase. However, nowadays, RP chromatography with aqueous mobile phase is by far most commonly used. RP chromatography is suitable for polar analytes, such as illicit drugs. The mobile phases commonly consist of a mixture of water and organic solvent, typically acetonitrile or methanol. Mobile phase buffers are used in RP chromatography in order to control the ionization of the analytes and improve chromatographic performance. However, when LC is combined with MS detection the type of buffer has to be considered. Some traditional non-volatile buffers should be avoided as they can precipitate in the ion source causing a decrease in signal intensity [Kronstrand and Josefsson 2006]. In LC-MS, specific mobile-phase additives are commonly added to influence the ionization characteristics e.g. small organic acids like formic acid and acetic acid are added to the mobile phase to improve the ionization of the compounds and consequently the sensitivity [Niessen *et al.* 2006a, Niessen 2006b]. In some cases other additives, such as ammonium formate or acetate, are also used to benefit chromatographic separations in terms of retention, resolution and/or peak shape.

High-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) have been used for the determination of licit and illicit drugs and their metabolites. In general, chromatographic separation has been carried out on

RP columns (C_{18}). In the last few years, conventional HPLC has been replaced more and more by UHPLC due to the advantages of using this technique. By using shorter columns (5 -10 cm) and smaller particle size (< 2 µm diameter against 3 - 5 µm diameter for conventional HPLC), and with the advancements in bridging structure of the column packing, UHPLC provides increased resolution and shorter run time, important parameters in terms of sample throughput and sensitivity improvement. Moreover, the gain in separation efficiency minimizes component co-elution, *i.e.* minimization of matrix interferences, and renders high mass spectra purity, improving screening processes [Ibáñez *et al.* 2008]. However, because of the narrowness of chromatographic peaks, this technique requires the use of MS instruments with fast scanning rates *i.e.* low dwell times, as well as LC pumps capable of standing the high column back pressure generated by the system (up to 15000 psi / 1000 bar).

The ***interface*** is an important part of an LC-MS system, located between the LC (liquid phase) outlet and the MS analyzer (gas phase under high vacuum). The most frequently used are atmospheric pressure ionization (API) interfaces: electrospray ionization (ESI) (**Fig. 1.6**) and atmospheric pressure chemical ionization (APCI). ESI allows the analysis of thermolabile compounds of a wide range of molecular weight and is considered most suitable for polar or even ionic compounds, whereas the APCI offers better results for less polar analytes. Both interfaces can work in either positive ionization mode (commonly $[M+H]^+$, where a molecule receives a proton (basic molecules)), or in negative ionization mode (commonly $[M-H]^-$, where a molecule releases a proton (acidic molecules)). Working in positive ionization the formation of sodium, potassium or ammonium adducts can also occur. The selection of ESI versus APCI, and positive versus negative ionization mode, depends on both the physical-chemical characteristics of the analytes and the sample matrix.

The interface that has been almost exclusively used for the determination of IDs and metabolites is electrospray. ESI is considered to be more affected by matrix interferences than APCI, causing more suppression or enhancement of the ionization

signal of analytes. In spite of this, ESI seems more effective for IDs allowing better analytical performances, especially for the more polar compounds.

When using LC-MS with an ESI interface, the flow of the mobile phase, in which the analytes are dissolved, passes through a capillary at atmospheric pressure set at an elevated voltage (typically 3-4kV). This high voltage disperses the liquid flow, forming a fine aerosol of charged droplets (nebulization), which are traversed through the atmospheric pressure region of the source to the MS. During this traverse the evaporation of the solvent (desolvation) from the charged droplets is assisted by a flow of heated gas, generally nitrogen. The solvent evaporates from a charged droplet until it becomes unstable upon reaching its Rayleigh limit. At this point, the droplet deforms as the electrostatic repulsion of like charges becomes more powerful than the surface tension holding the droplet together. Then, it undergoes Coulomb fission, whereby the original droplet 'explodes' creating many smaller, more stable droplets. The new droplets suffer further desolvation and subsequently Coulomb fissions until the final production of gas-phase ions, leaving the analytes with the charges that the droplet carried. This theory is known as the "charge residue model". Finally, the ions formed are directed into the first vacuum stage of the MS by focal lenses.

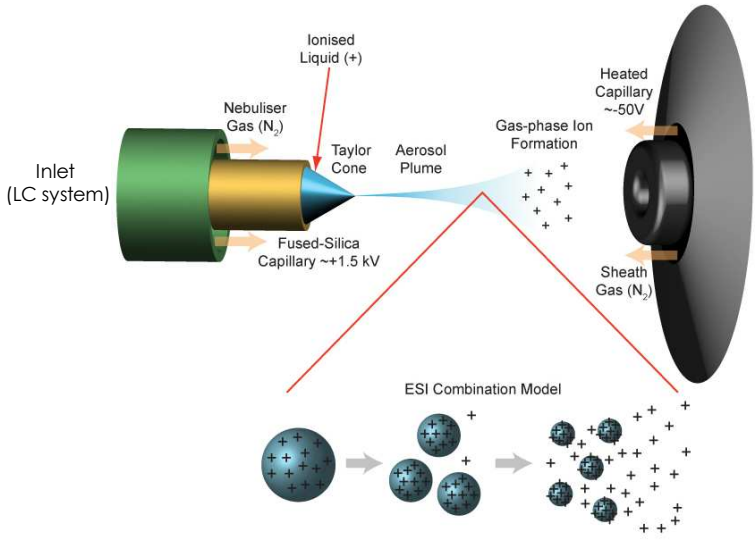


Figure 1.6. Schematic overview ESI [http://www.lamondlab.com/MSResource/LCMS/MassSpectrometry/electrosprayIonisation.php]

Mass spectrometry (MS) is an analytical technique that measures the molecular masses of individual compounds. The interface converts components of a sample into rapidly moving gaseous ions and by separating these charged ions on the basis of their mass-to-charge (*m*/z) ratios, either in time or space, a mass spectrum is obtained. The different mass spectrometers can be distinguished by the type of mass analyzer, each with their specific strengths and drawbacks. Some popular designs are quadrupole (Q), ion trap (IT), time-of-flight (TOF) and Orbitrap. The mass analyzer is considered to be the heart of the mass spectrometer and its performance is characterized by several parameters, typically resolution (R) (*i.e.* the ability to distinguish two peaks of slightly different *m*/z values) and mass accuracy (*i.e.* the uncertainty in the measured *m*/z ratio), but also mass range, efficiency, linear dynamic range, speed and sensitivity. The choice of instrument depends mainly on the application, adaptability and costs.

Mass spectrometry is the most widely applicable and powerful analytical tool nowadays available for the qualitative and quantitative determination of most organic compounds in complex matrices. The combination of different mass analyzers has evolved to achieve tandem (MS/MS) instruments, which have improved sensitivity and selectivity and/or provide a great amount of information for identification and elucidation purposes.

All the work performed in the present doctoral thesis is based on tandem mass spectrometry (MS/MS), using the following configurations: triple quadrupole (QqQ), hybrid quadrupole time-of-flight (QTOF) and linear ion trap Orbitrap. These tandem mass instruments will briefly be discussed below. A more detailed and profound discussion on mass spectrometry and the different mass analyzers can be found in the literature [Niessen 2006b; Dass 2007].

A ***triple quadrupole mass analyzer (QqQ)*** consists of two quadrupole mass analyzers (Q) in series, with a non-mass resolving/filtering hexapole (q) usually in between, which acts as a collision cell. The collision cell uses an inert gas (generally argon) to provoke collision between the gas molecules and an ion that is selected in the first quadrupole

Q_1 (precursor ion) to produce fragment ions (product ions). This process is known as collision-induced dissociation (CID). Subsequently, fragment ions are passed through the second quadrupole Q_2 where they may be filtered or scanned.

Each quadrupole (Q_1 and Q_2) can work in either Full Scan or Selected Ion Monitoring (SIM) mode. Accordingly, multiple acquisition modes can be carried out when using triple quadrupole analyzers. The selection of the mode depends on the objective of the analysis. In continue, the four main modes will briefly be explained:

Product ion scan (or daughter scan): Q_1 is set to isolate an ion of a known *m*/z (precursor ion), which is fragmented in the collision cell (q). The Q_2 is then set to scan within a given *m*/z range, giving information on the product ions generated from the selected precursor ion. The information obtained from ion fragmentation can be useful for the elucidation of the structure of the original ion, although this working mode is commonly performed to identify the transitions used (precursor ion → product ion) for a subsequent analysis (quantification and confirmation) by tandem MS.

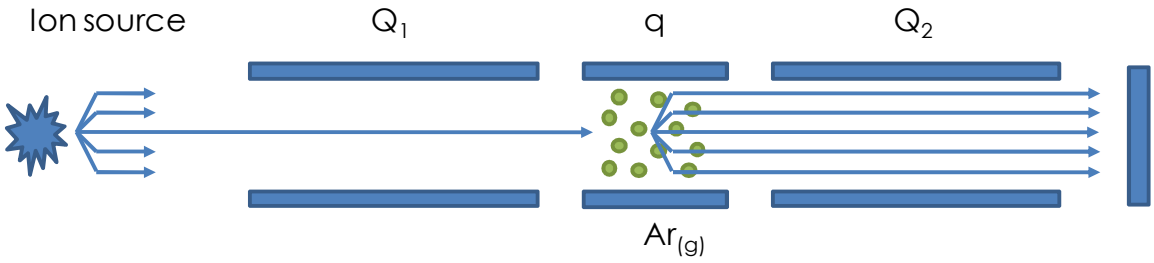


Figure 1.7. A schematic overview of product ion scan

Precursor ion scan (or parent scan): A product ion is selected in Q_2, and the precursor masses are scanned in Q_1. This experiment is selective for ions having a particular functional group (*e.g.*, a phenyl group) released by the fragmentation in q. It might also

be useful to obtain more insight in fragmentation pathways of certain compounds [Bijlsma *et al.* 2011 – *scientific article 6*].

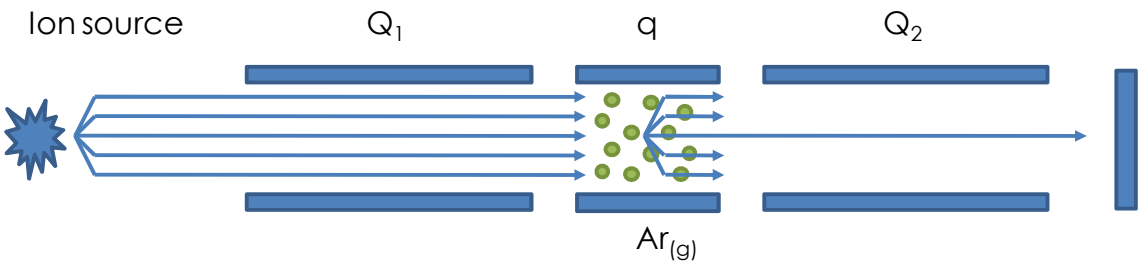


Figure 1.8. A schematic overview of precursor ion scan

Neutral loss scan: Both Q_1 and Q_2 are scanned together, but with a constant mass offset. This allows the selective recognition of all ions which, by fragmentation in q, lead to the loss of a given neutral fragment (*e.g.*, H_2O, CO, NH_3, benzoic acid). Similar to the precursor ion scan, this technique is also useful in the selective identification of closely related class of compounds in a mixture or unknown sample.

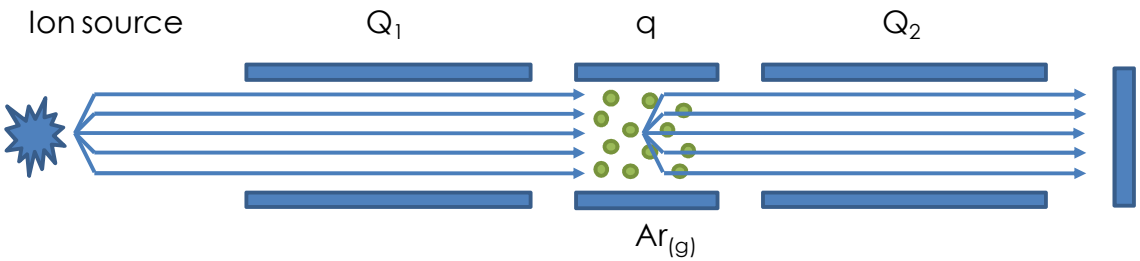


Figure 1.9. A schematic overview of neutral loss scan

Selected reaction monitoring (SRM): In this mode a specific transition is monitored, as both Q_1 and Q_2 are set to a selected *m/z*, allowing only a certain product ion from a certain precursor ion to be detected.

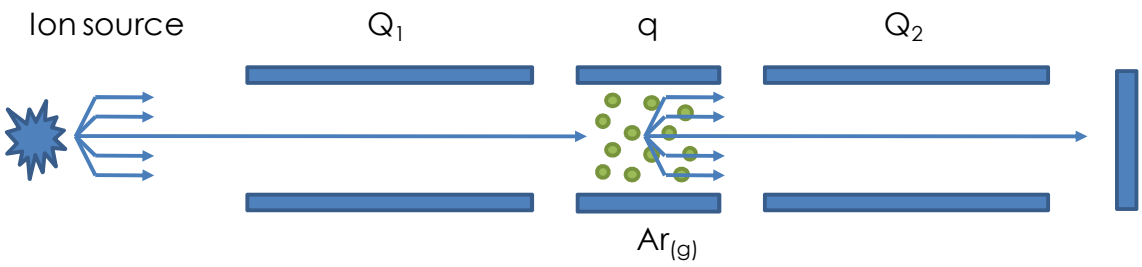


Figure 1.10. A schematic overview of selected reaction monitoring

SRM is a highly selective and sensitive way of performing an analytical determination, since the background noise is low, improving the signal-to-noise (S/N) ratio. In addition, several specific transitions can be measured during the same analysis permitting the simultaneous determination of different compounds (*i.e.* multi-residue analysis) and their reliable confirmation. For these reasons, LC-MS/MS with triple quadrupole analyzers is commonly employed under SRM mode. It is highly appreciated in quantitative analysis in terms of selectivity, sensitivity and robustness. Its application to qualitative analysis is however limited due to the relative low resolution (approximately 1 Da) and low sensitivity in full scan mode.

In this thesis, LC-MS/MS under SRM mode has been used, as it shows excellent characteristics for quantification of target IDs and metabolites at low concentrations in complex water samples, like influent or effluent wastewaters. The scan mode options have been used for the optimization of the MS method in relation to instrumental parameters (*i.e.* selection of product ions, cone energy and collision energy) and in some particular cases in order to identify the precursor ions of certain fragment ions.

Hybrid quadrupole-time of flight mass analyzer is based on the time of flight (TOF) technology. The sensitive full spectrum acquisition and high speed capabilities, the high mass resolving power and mass accuracy provided by TOF MS instruments makes this technique very attractive and suited for identification and wide-scope screening purposes. TOF mass analyzers consist of a flight tube in which ions are separated on the basis of their velocity differences. Ions are accelerated by an energy pulse. The velocity of the ion depends on the *m*/*z* ratio. The time that is necessary to reach a detector at a known distance is measured and the flight time for each ion of particular *m*/*z* is unique *i.e.* heavier ions reach lower speeds than lighter ones. The reflectron lenses focus all ions with the same *m*/*z* value, so they arrive simultaneously at the detector. The result is an improvement in mass resolution and allows measuring ions at accurate mass. The latest generation of TOF MS instruments provide high mass accuracy, commonly below 3 ppm, and mass resolving power higher than 40,000 full width at half maximum (FWHM).

Hybrid quadrupole time of flight mass spectrometry (QTOF MS, **Fig. 1.11**) offers more possibilities in screening and identification. The use of QTOF MS permits, like a single TOF, the acquisition of full-scan spectra providing the accurate mass of (de)protonated molecules, but it can also provide relevant structural information by obtaining product ion full-scan spectra at accurate mass. The latter is possible when performing MS/MS experiments, an excellent approach to give extra confidence to confirm or deny a suggested structure. However, pre-selection of the precursor ion in MS/MS experiments may limit the potential of QTOF MS for wide-scope screening purposes, as other possible contaminants not included as target analytes in the method might be ignored (non detected), even if they are present at high levels in the sample. An interesting feature of some QTOF systems (e.g. from Waters, used in this thesis) is the possibility to acquire in MS^E mode, where the quadrupole works as an ion guide and fragmentation is promoted in the collision cell. The simultaneous acquisition of accurate-mass full-spectra at low and high collision energies provides useful information on the (de)protonated molecules (low energy, where low fragmentation occurs) and product ions (high energy, where fragmentation is promoted) from a single experiment without the need of pre-selecting the analytes.

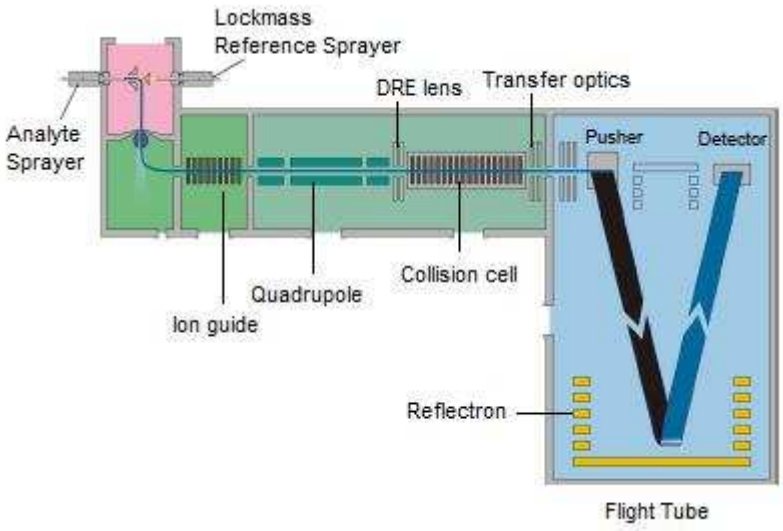


Figure 1.11. Schematic diagram of a hybrid QTOF mass spectrometer [http://www.vtpup.cz/common/manual/PrF_kach_Waters_Q-TOF_manual_EN.pdf]

A schematic diagram of a ***linear ion trap Orbitrap mass analyzer*** is shown in **Figure 1.12**. This hybrid mass spectrometer combines both a linear ion trap (LTQ) and a Fourier transform (FT) Orbitrap mass analyzer. Ions generated in the ion source are trapped in the LTQ, which can be used as a selective mass filter in order to perform MS^n scan mode experiments. Then, the product ions are axially ejected from the LTQ and collected in a C-shaped ion trap (C-Trap). Stored ions are pulsed out of the C-Trap orthogonally and as small packets into the Orbitrap. Conversely, the ions are directly ejected from the LTQ and subsequently the C-Trap and pulsed into the Orbitrap to allow full scan acquisition. An Orbitrap consists of an outer barrel-like electrode and a coaxial inner spindle-like electrode that form an electrostatic field. Trapped ions undergo rotation around the inner electrode and harmonic oscillation along its axis. The *m*/z values are measured from the frequency of harmonic ion oscillations. Orbitrap mass analyzers provide high mass accuracy, (typically 2 - 5 ppm), high resolving power (up to100,000 FWHM), and dynamic range. A more detailed description can be found in the literature [Hu *et al.* 2005; Makarov *et al.* 2006]

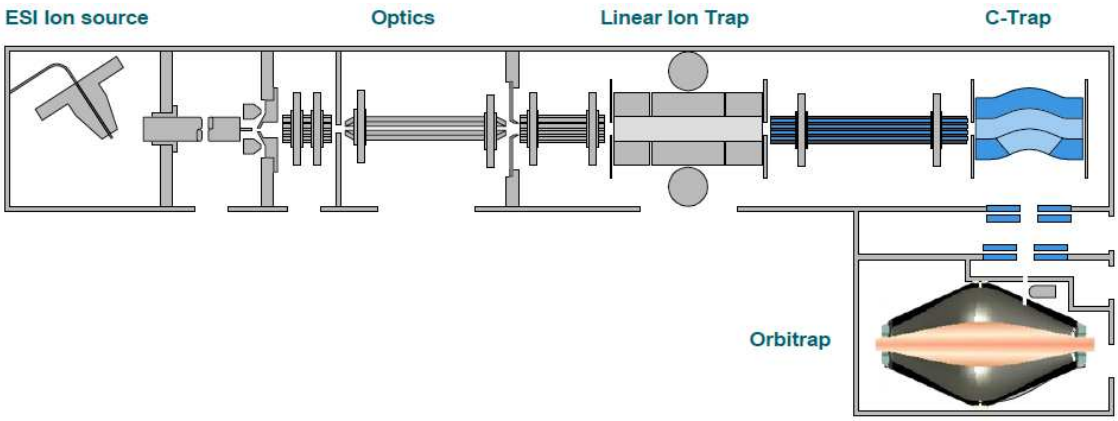


Figure 1.12. Schematic diagram of a hybrid LTQ FT Orbitrap mass spectrometer [Makarov *et al.* 2006]

The LTQ FT Orbitrap combines the sensitive full spectrum MS capabilities, high mass resolving power and mass accuracy of the Orbitrap analyzer with the tandem mass spectrometry capability of the linear ion trap. It permits the acquisition of full scan spectra measuring the accurate mass of (de)protonated molecules, and nominal mass measurements of corresponding product ions in the ion-trap (MS^n mode) in a single analysis. In this way, relevant information for identification and confirmation, e.g. retention time, molecular weight and fragmentation, can be simultaneously obtained.

1.3.3. Matrix effects in LC-MS analysis

Matrix effects are one of the main problems associated with analysis by LC-MS and LC-MS/MS. Matrix effects can affect both identification and quantification of analytes, especially at very low concentration levels.

Matrix effect is defined by IUPAC as "the combined effect of all components of the sample other than the analyte on the measurement of the quantity. If a specific component can be identified as causing an effect then this is referred to as interference [http://goldbook.iupac.org/M03759.html]". These interfering compounds co-elute with the compounds of interest altering the ionization process, leading to enhancement or (more frequently) suppression of the analyte signal. Hypotheses for the

occurrence of ion enhancement are rarely proposed, but suppression is likely induced by a competition between the interfering matrix compounds and the analytes in the formation of charged droplets as well as the amount of the analyte ions formed in the gas phase that reaches the detector. Matrix effects are notoriously variable in occurrence and intensity, and they depend on the physical and chemical properties of each analyte and the matrix itself, but also on other factors such as the methodology applied (*i.e.* sample preparation, chromatographic separation, mass spectrometry instrumentation and ionization conditions).

Matrix effects influence the method performance in terms of detection capability, selectivity, repeatability, accuracy and linearity, and can lead to considerable errors in the quantification of analytes [Gosetti *et al.* 2010]. For example, low and variable analyte recovery, resulting from the presence of matrix interferences, is one of the main factors affecting the sensitivity of established analytical methods [Kasprzyk-Hordern *et al.* 2007]. The evaluation of matrix effects in MS analysis should therefore be assessed and included in the method validation process, as well as the strategies to eliminate, minimize, or correct these effects. Matrix effects can be evaluated by comparing the response of the analytes in solvent with a post-extraction fortified sample extract. When dealing with complex environmental and wastewater samples, the complete elimination of matrix effects may not be feasible, thus effects should be minimized, besides that a compensation for matrix effects is needed for accurate quantification.

Several approaches can be applied to face matrix effects in quantitative multi-residue analysis, each having advantages and drawbacks [Hernandez *et al.* 2005a; Niessen *et al.* 2006a; Lehotay *et al.* 2010; Stahnke *et al.* 2012]. Some strategies used in pesticide residue analysis in food and feed, such as matrix-matched calibration or standard additions, [SANCO 2011] are less appropriate for the multiresidue analysis of IDs in water samples. The main reason is that it is difficult to obtain genuine blank samples, especially for influent wastewaters, that in addition is fully representative of the samples analysed (*i.e.* the same matrix composition). Furthermore, the presence of some compounds at trace concentration levels implies major errors when performing standard additions, an approach that is problematic as it requires the previous

knowledge of the approximate analyte concentration in the sample to adjust the additions, and that is normally subjected to high errors when extrapolating the calibration to obtain the analyte concentration in the sample.

For the determination of IDs and metabolites in complex water samples other approaches are commonly applied to compensate for matrix effects. One of them is the application of an adequate/ selective ***sample pretreatment*** *i.e.* the removal of interfering components. As previously described, off-line SPE is widely applied for the determination of IDs in aquatic samples. Although SPE can perform some sample clean-up, it is mainly used for analyte pre-concentration purposes. However, it may also lead to a pre-concentration of interfering components, generating the opposite of the desired effect. Alternative procedures such as on-line SPE and large volume injection (LVI) have been proposed for sample preparation [Berset *et al.* 2010; Chiaia *et al.* 2008; Postigo *et al.* 2008]. However, these procedures do not normally minimize matrix effects and the benefits are particularly to reduce time and/or cost, and to improve sample throughput.

It has been reported in the case of multi-residue methods for complex matrices, that no sample preparation method can remove co-eluting interfering components completely enough without reducing recoveries of at least some analytes [Lehotay *et al.* 2010].

A simple strategy to reduce matrix effects is ***sample dilution*** or to use ***smaller injection volumes***. In this way, the amount of matrix introduced in the ion source is reduced, thereby decreasing signal enhancement/suppression problems generated by interferences. Typically, by introducing less sample volume, the limit of detection (LOD) and limit of quantification (LOQ) increase. However, detectability of the analytes may improve by sample dilution if the undiluted samples/extracts overload the system [Lehotay *et al.* 2010]. In addition, new generation analytical instruments allow significant reduction of matrix load without evidence of sensitivity loss [Martínez-Bueno *et al.* 2011]. A moderated sample dilution may be beneficial in two ways (1) improvement of the chromatographic performance of the analysis; (2) less amount of

sample load may even lead to an increased sensitivity. Dilution or smaller injection volumes might therefore be beneficial depending on the analyte / matrix combination, and on the analytical instrument used.

The preferred approach to compensate for matrix effects is the use of ***internal standards***. The best analytical procedure is to add internal standards as surrogates, prior to any sample treatment. The ideal internal standard would be in the same way affected by potential losses associated to the sample treatment, and by matrix interferences as the target analyte. Typically isotope-labelled internal standards (ILIS) of the corresponding analyte are used to this aim. ILIS are preferably ^{13}C and ^{2}H (deuterated) labelled compounds, as their natural abundance are very low (approx. 1.1% and 0.01%, respectively). An advantage of using ILIS is that they are not present in the aquatic environment and thus getting a representative genuine blank sample would not be a problem in this case.

Although the use of ILIS is one of the best approaches for matrix effects correction, its application gets complicated in multi-residue multiclass methods where a large number of ILIS would be required. ILIS are usually expensive and analyte-own ILIS are sometimes limited in commercial availability e.g. for new drugs or legal highs. Therefore, ILIS are frequently shared to correct for several compounds. Normally ILIS with the closest structure, or with the closest retention time is selected for correction when analyte ILIS is not available. However, the use of analogue compounds as internal standards does not always ensure satisfactory correction [Marín *et al.* 2009]. When ILIS are unavailable, another way for reduction, or at least minimization of matrix effects, needs to be applied (*e.g.* an additional or more extensive clean-up step, adjustment of LC or MS conditions, etc). In general, even when analyte-own ILIS are available, a reduction of matrix effects is recommended for better precision, sensitivity and robustness in complex matrix samples.

1.3.4. Quality criteria in LC-MS analysis

Any analytical methodology should comply with several quality requirements, in order to generate reliable data for accurate quantification and safe identification. Method

validation is obviously required, and the application of realistic criteria based on the acquisition of several MS/MS transitions considering their specificity, or criteria based on mass measurement accuracy is also necessary. Thus, the accomplishment of retention time and ion intensity ratios when compared with the reference standard is mandatory for reliable identification of the compound. This is of particular importance due to the absence of detailed guidelines in the field of environmental analytical chemistry. Quality procedures applied by researchers in other fields of investigation (mainly food-safety) are normally taken and adapted in the environmental field.

Quality controls, both internal and external, are required to ensure the quality of the analysis, and are key aspects involved in quality assurance and in the application of appropriate analytical methodologies.

Quantification. An analytical methodology needs to be fully validated to ensure the quality of the results. Several parameters are defined by the SANCO guidelines to evaluate the overall analytical procedure [SANCO 2011]. Although these guidelines apply to pesticide residue analysis, they are highly useful and may be adopted in other fields. Based on these guidelines, the performance of the methods should be validated in terms of linearity, LOQ, LOD, accuracy and precision. Validation should be performed prior to the analysis of samples at analyte concentrations close to the expected concentrations in real samples, since matrix effects may depend on the concentration of the analyte [Liang *et al.* 2003].

Due to the difficulty of applying matrix-matched standard calibrations in the environmental field, the use of the analyte-own ILIS has mostly been applied for accurate quantification of each target analyte. ILIS are added to compensate for matrix effects and, if used as surrogates, they facilitate the satisfactory correction for potential analytical errors associated to sample manipulation and storage.

Another issue necessary to test for daily method variations and method robustness is the use of internal quality controls (QCs). To each sample batch (max. 10 samples), one sample QC is normally included. A blank sample, free of any drugs, is difficult to obtain

in some fields, e.g. influent wastewaters. Therefore, in these particular cases, a sample with lowest concentrations expected might be selected for this aim. The QC ("blank" sample spiked at realistic analyte concentrations) together with the non-spiked "blank" sample should be analyzed within the same batch. The concentration values found in the non-spiked sample should be subtracted from the QC to obtain recoveries. In this way, a better insight into the data would be obtained and possible errors might be explained (e.g. non-compliance of confirmation criteria might occur due to matrix interferences). In light of realistic data obtained for QCs, the sample sequence can be considered as satisfactory, typically when recoveries range from 60 to 130% for the IDs detected in sewage water.

Inter-laboratory comparison exercises are also important for regular checks of quality control, including the assessment of accuracy and possible biased errors. In this PhD thesis, external QCs were analyzed using the developed methods. The external QCs formed part of inter-laboratory comparison exercises carried out in 2012 and in 2013, which were organized and coordinated by the Sewage Analysis CORe group Europe (SCORE) network (see also Chapters 5 and 6). The data of each participating laboratory were tested for the normality of the distributions (Shapiro-Wilk test) and presence of outliers (Grubbs or Veglia), and z-scores were evaluated. Z-scores are an accepted measure for the performance of an individual laboratory with regard to the group average [UNIDO 2006]. The variability in the mean of laboratories provides an indication of how closely laboratory results match each other at a particular concentration. The results may disclose systematic errors or poor performance in individual laboratories.

Confirmation of the identity. Confirmation of analyte identity is necessary to avoid reporting false positives and/or false negatives. Analytical chemists have to develop reliable methods that allow not only accurate quantification of targeted analytes but also, and even more importantly, their unequivocal identification. Ideally, confirmation of the identity should be objective and safe, not depending on the subjective

interpretation of the analyst, so predefined, efficient confirmation rules are necessary [Pozo *et al.* 2006].

Low resolution (LR) MS/MS systems typically operate under SRM mode, where a precursor ion is selected in the first quadrupole, fragmented in the collision cell, and a "specific" product ion is selected using the second quadrupole. The selection of a specific SRM transition, avoiding the use of common losses (e.g. H_2O, CO_2 and HCl), is important in order to prevent the reporting of false positives or false negatives (Pozo *et al.* 2006). However, even with this approach, there are some probabilities that other compounds, not related to the analyte, can share the same transition. Therefore, a second SRM transition is often monitored and the presence of a compound is only considered to be confirmed if both transitions produce chromatographic peaks with retention times corresponding to that of the investigated analyte in pure standard. In addition, the ratio of the intensities of the two recorded SRM transitions must be similar to that obtained for the reference standard. This concept has been further refined in the European Commision Decision (2002/657/EC), which regulates the requirements for analytical methods used to quantify and confirm organic residues (e.g. veterinary drugs) in live animals and animal products, and in the SANCO/12495/2011, which regulates pesticide residue analysis in food and feed. These guidelines are often used as a reference for researchers working in the field of environmental analytical chemistry.

HRMS, such as time-of-flight and Orbitrap, has also been used for quantification and confirmation of IDs using sensitive full-scan accurate mass data. In the more recent SANCO/12495/2011 document, a criterion for high resolution mass spectrometry (e.g. time-of-flight and orbitrap) is defined. Here, the quality of information provided by HRMS is based on mass accuracy measurement rather than on resolution power.

The identification/confirmation criteria mentioned above are the most frequently used by analytical chemists to develop reliable analytical methods. Essentially these criteria are based on the collection of identification points (IPs). IPs are earned depending on

the mass analyzer used, distinguishing between low resolution and high resolution instruments, and between MS and MS^n. A minimum of 4 IPs is recommended (**Table 1.1**).

Table 1.1: The relationship between a range of classes of mass fragment and identification points earned [2002/657/EC].

MS technique	Identification points earned per ion
LRMS	1.0
LR-MS^n precursor ion	1.0
LR-MS^n transition product	1.5
HRMS	2.0
HR-MS^n precursor ion	2.0
HR-MS^n transition product	2.5

In order to correctly identify a compound the relative ion intensities between both recorded transitions (Q/q ratio) need also to comply. This ion ratio should coincide with the ion ratio obtained from the reference standard, in accordance with maximum permitted tolerances (**Table 1.2**). In addition, the maximal accepted deviation in retention time is 2.5 %, between the compound in sample and the reference standard.

Table 1.2: Maximum permitted tolerances for relative ion intensities [2002/657/EC; SANCO/12495/2011].

Relative intensity (% of base peak)	Q/q ratio value	Tolerance (%)
> 50%	1 – 2	20
> 20 to 50%	2 – 5	25
> 10 to 20%	5 – 10	30
≤ 10%	≥ 10	50

Note: the Q/q ratio of the reference standard should be calculated with the standards included in every sequence of sample analysis.

These tables have been widely used by researchers. Recently there has been a modification in the SANCO guideline [SANCO/12571/2013], which recommend using the same maximum tolerance (± 30%) independently on the ion ratios. It is also recommended not to take the tolerances as absolute limits without complementary interpretation by an experienced analyst.

As previously stated, the reliable determination of IDs in the aquatic environment (both surface water and wastewater) is an analytical challenge, owing to the low concentrations of these compounds and the complexity of the samples. The proposed quality procedures, recommended by scientist from other research field, might not always be realistic in sewage water sample analysis. Therefore, more details and recommendations on how to proceed when certain quality criteria are not accomplished will be given in chapter 5 of this Thesis.

1.4 Scientific articles included in this PhD thesis

The core of this doctoral thesis consists of 1 book chapter and 9 scientific articles published in peer-reviewed journals within the first quartile of the ISIS web of knowledge. In addition, 3 scientific articles are included, which are the result of the collaboration within a European-wide international network (Chapter 6). It is important to emphasise that several of articles included in this doctoral thesis have been made in close collaboration with acknowledged researchers who pertain to well-known and prestigious international research institutes. The role of the PhD candidate within these collaborative studies will be explained below:

Scientific articles 5 and *9* result from a collaboration with KWR Watercycle Research Institute (Nieuwegein, the Netherlands). The contribution of the PhD candidate to these manuscripts consisted of designing the studies. He coordinated sampling and validated the analytical protocol in order to subsequently employ it for analysis. Furthermore he compiled and evaluated data, drafted and revised the manuscripts. This research has been performed independently under the supervision of Prof. Dr. Pim de Voogt and Prof. Dr. Félix Hernández.

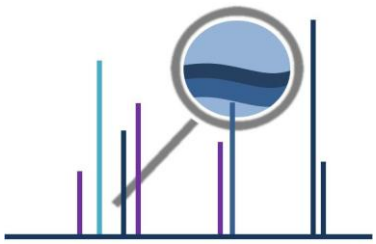
Scientific articles 6 and *7* result from a close collaboration between the PhD candidate and Prof. Dr. Wilfried Niessen, a recognised specialist on mass spectrometry, of hyphen MassSpec (Leiden, the Netherlands). The experimental work was performed at the University Jaume I and the PhD candidate designed the study, performed the experiments and analysis, compiled and evaluated data, drafted and revised the manuscripts.

Scientific article 10 is the result of a collaborative study between the National Institute for Public Health and the Environment (RIVM), KWR Watercylce Research Institute, University of Antwerp and the University Jaume I. The PhD candidate performed the analysis of water samples based on the analytical protocol that he previously developed and validated (*scientific article 1* - Bijlsma et al. JCA 2009). He also helped with the development and validation of an analytical protocol for RIVM, and contributed to the draft manuscript and the subsequent reviewing. This research has

been performed independently under the supervision of Dr. Bert Baumann, Ir. Ans Versteegh and Prof. Dr. Félix Hernández.

The previous work also resulted in a RIVM Report 703719064/2010 "Drugs of abuse and tranquilizers in Dutch surface waters, drinking water and wastewater" coordinated by the RIVM. The investigation took place by order and for the account of VROM-Inspectorate, within the framework of the Programme for Clean and Safe Water, project 703719 Monitoring and Enforcement Drinking Water Act. KWR Watercycle Research Institute, which participated in this study, received financial support from the Joint Research Programme (BTO) of the Dutch water companies. This report is also registrated as BTO 2011.023. The digital version of the RIVM report is available on the website of the RIVM (www.rivm.nl). [http://www.rivm.nl/dsresource?objectid=rivmp:26501&type=org&disposition=inline&ns_nc=1].

The three articles included in Chapter 6 *(scientific articles a, b and c)* are the product of a European-wide international network and do not form the core of this Doctoral thesis. The role of the PhD candidate was focused on providing data from wastewater samples of Castellón (Spain) and Germany. He also actively participated in writing some sections of the papers. More details on the work done will be given in Chapter 6. In addition to the three articles, a chapter is written for a monograph on SBE coordinated by the EMCDDA (*scientific monograph, chapter 2)*. This report is an update on a previously published monograph "EMCDDA insight 9: Assessing illicit drugs in wastewater: Potential and limitation of a new monitoring approach".



CHAPTER 2

Development of multi-residue methods for the determination of illicit drugs in water using UHPLC-MS/MS with triple quadrupole mass analyzer

2.1 Introduction

The principal difficulty when developing a multi-residue method for the determination of IDs and their metabolites in the aquatic environmental deals with the low analyte concentrations, the high variability in the physic-chemical characteristics of the target analytes and the complexity of the sample matrix (especially wastewater samples). Analytical procedures normally consist of different steps in order to achieve the required selectivity and sensitivity. Nowadays, the analytical technique of choice in this field is LC-MS/MS, with triple quadrupole or iontrap mass analyzer.

The physical and chemical composition of water, especially sewage water, is rather complex. The removal of matrix components that may potentially interfere and compete with the target analytes in the ionization process in LC-MS/MS analysis is crucial. Off-line SPE is widely applied for sample clean-up, but mainly for analyte pre-concentration. Prior to SPE, samples are filtered (typically GFC 0.45 μm) or centrifuged to remove suspended particulates and to prevent SPE material from clogging. The SPE cartridges used for the analysis of IDs are typically reversed phase polymeric cartridges, most commonly Oasis HLB. Owing to the basic character of many consumed IDs, Oasis MCX is also an interesting sorbent, as pH and polarity can be changed during loading, washing and elution steps improving the selectivity towards basic analytes. However,

the hydrophilic/lipophilic character of the more generic Oasis HLB sorbent makes it suitable to retain a high variety of analytes of different polarities and properties (acid, neutral, basic). This allows widening the scope of the method and surely for this reason is the cartridge most often selected at present.

In spite of the advantages of SPE, this approach also has some limitations such as low and variable analyte recovery, pre-concentration of matrix interferences resulting in signal enhancement or suppression, and the time and/or costs involved. Alternative sample treatment approaches for the determination of IDs in sewage, such as SPE in on-line mode [Postigo *et al.* 2008] and large volume injection [Berset *et al*. 2010; Chiaia *et al.* 2008; Martinez-Bueno *et al.* 2012] open the possibility for a full-automated analysis, minimizing sample manipulation and obtaining a shorter time-frame for both clean-up and analysis. Furthermore, they allow reducing the amounts of solvents, waste and costs of analysis. Conversely, these methods may suffer from lower sensitivity (because less sample volume is commonly loaded), strong matrix effects and/or worse chromatographic performances [Bagnati and Davoli. 2011]. In the near future, new and more sensitive LC-MS instruments may help to improve the performances of these alternative procedures.

Considering the matrix, but even more, the polar character of most analytes, the use of LC as separation technique for the determination of IDs in the aquatic environment seems rather logical. LC is a more versatile technique with minor sample treatment and shorter run times compared to GC and it is highly appropriate for polar/non-volatile analytes. This is reflected by the fact that most of the methods recently developed for IDs in environmental samples are based on LC coupled to MS/MS. Both HPLC and UHPLC have been used to separate targeted IDs and their metabolites. Compared to conventional HPLC, UHPLC provide better chromatographic resolution *i.e.* narrower chromatographic peaks (generally 2 - 7 seconds), improved sensitivity, and shorter chromatographic runs, and consequently higher sample throughput. UHPLC is based on the use of columns with a particle size < 2 μm, which allow less diffusion of the molecules over the stationary phase, and able to withstand high flow rates and accordingly high pressure. However, to take full advantage of UHPLC separations a

fast-acquisition triple quadruple mass analyzer is needed, capable of working at low dwell times *i.e.* the time for which a mass or transition is monitored. This can be achieved by a "travelling wave" or moving electric field, a modification in the collision cell, which allows reducing the residence time of ions and the level of cross-talk between successive transitions. In this way, modern mass analyzers can take profit of the increased resolution of UHPLC providing a minimum of 10 data points across each chromatographic peak without evident sensitivity losses.

LC separation of IDs and metabolites has mostly been performed using reversed-phase (C_{18}) columns, with a moderately polar mobile phase consisting of a mixture of water and organic solvent (typically acetonitrile or methanol) [van Nuijs *et al.* 2011a]. Specific mobile-phase additives have been incorporated to benefit chromatographic separations and/or to influence the ionization characteristics, as the mobile-phase composition can play an important role in electrospray nebulisation and ionisation process [Niessen 2006b]. Generally, the aqueous mobile phases consisted of water, ammonium formate or ammonium acetate (1-50 mM) and have usually been acidified with formic or acetic acid (0.05 – 0.1 %) to improve the ionization of IDs, which are normally best ionized in positive ion mode. However in the case of cannabis, which are frequently determined under negative ion mode, no additives [Postigo *et al.* 2008], a basic solution of triethylamine [Castiglioni *et al.* 2006] or water with 0.05% ammonium formate [Hogenboom *et al.* 2009] have been used.

Ionization of IDs and their metabolites has been mainly performed using ESI, as this source seems more effective and allows better analytical performances for this type of compounds. However, ESI is considered to be more prone to matrix interferences than other ion sources (e.g. APCI), leading to more suppression or enhancement of the analyte ionization signal. This is particularly evident with complex-matrix samples, such as influent wastewaters. Thus, in addition to the sample extraction process, most of the reported methodologies have applied ILIS for matrix effects correction in order to get accurate quantification. The own analyte ILIS is generally used to compensate for matrix effects in wastewater matrices, and also for possible errors related to sample pre-

treatment and even degradation during storage when ILIS are added to the samples as surrogates at the sample storage (i.e. before SPE step).

As stated above, LC-MS/MS with triple quadrupole mass analyzer is nowadays the workhorse in this field in order to get the reliable quantification and confirmation of IDs, at low concentration level (ng/L), in complex water samples. This low resolution MS system typically operates in SRM mode, which requires the previous selection of analytes before measurement. This (pre-)target analysis implies the optimization of MS/MS conditions for each compound of interest. MS/MS compound-dependent parameters, such as cone voltage, collision energy, are optimized by means of infusion experiments, *i.e.* direct infusion of individual standard solutions. Firstly, the ionization mode and cone voltage are established to define the precursor ion. In continue, after isolation of the precursor ion, different collision energies are set to obtain abundant and specific product ions. Accordingly, various SRM transitions (precursor ion > product ion) can be monitored, taking into account the sensitivity/specificity of each transition. Typically the most abundant SRM transition is selected for quantification, but it is important to stress that not only the abundance or intensity of the products ion should be taken in to account, but also its quality. In order to minimize matrix interferences, specific transitions should be selected if possible, as acquiring non-specific transitions might lead to report false positives or even false negatives, because of the non-compliance of the ion ratios. For this reason, product ions corresponding to common losses such as water, carbon monoxide, formic acid, etc should be avoided.

Basically, the acquisition of two specific transitions should serve for a reliable quantification and confirmation of the presence of a compound [2002/657/EC; SANCO/12495/2011]. The concept of collecting IPs, as previously discussed in the introduction, is useful when dealing with confirmation issues: 1 IP is earned by the precursor ion and 2 x 1.5 IP by each product ion, when using low resolution triple quadrupole instruments (4 IPs in total). Most of the developed methods for the analysis of IDs in environmental samples meet these criteria. However, a critical confirmation parameter, the ion ratio between both recorded transitions and the maximal accepted deviation in relative retention time, is often unreported or not sufficiently discussed.

2.2 Scientific articles

One of the primary objectives of this doctoral thesis was the development of sensitive analytical methods that provide confident detection and quantification of IDs often found at very low concentration levels in the aquatic environment. In this chapter, two studies using UHPLC-MS/MS with a fast acquisition triple quadrupole mass analyzer are discussed. Prior to analysis by LC-MS/MS, both developed methodologies applied off-line SPE for clean-up and analyte concentration. It is worth mentioning that each compound, except norBE and norcocaine, was quantified using its corresponding ILIS, which was used as surrogate to ensure the satisfactory correction for potential analytical errors associated to sample preparation, storage and matrix effects. Furthermore, special emphasis was placed on the reliable confirmation of IDs detected in water. In these works the acquisition of three SRM transitions per compound facilitated the simultaneous detection, quantification and confirmation of positives samples.

The first work (*scientific article 1*) presents the development, validation and application of an advanced analytical methodology for the simultaneous quantification and confirmation of 11 basic/acidic IDs and relevant metabolites in surface water and urban wastewater. The IDs selected included several amphetamines, cocaine and its metabolites, and the main metabolite of cannabis. The sample pre-treatment consisted of an off-line SPE procedure using Oasis MCX cartridges. MCX is a strong cation-exchange mixed mode polymeric sorbent build upon HLB copolymers that allows improved selectivity towards basic analytes. Special effort was done to optimize sample preparation and LC separation to incorporate THC-COOH in this method. After sample treatment, the selected drugs were separated with a BEH C_{18} column (50 mm x 2.1 mm I.D.) within 6 minutes under UHPLC optimized conditions. BEH stands for *Ethylene Bridges Hybrid* and consist of small particles, size 1.7 μm, which combine inorganic (silane) and organic (polymere) properties. The use of UHPLC in this study is worth highlighting as in the year of publication (2009) few articles were published using UHPLC-MS/MS for the determination of IDs in water samples. The combination of UHPLC together with a fast MS/MS (TQD analyzer from Waters) allowed a rapid, sensitive and confirmative

determination of the selected analytes. Regarding quantification, matrix effects were studied in different water types and the use of analyte deuterated compounds as ILIS for proper correction was also evaluated. The developed method was successfully and routinely applied at our laboratory for several years. The manuscript has received much attention from the scientific literature and has been highly cited. It, therefore, received the Elsevier 2010 award as being one of the top 50 articles with more citations in the period 2008-2009.

In a second study (*scientific article 2*) the methodology has been updated, using a more generic Oasis HLB SPE cartridge and taking advantage of the availability of a state-of-the-art UHPLC-MS/MS, in order to improve sensitivity and obtain lower limits of detection. A new UHPLC-MS/MS, with TQS analyzer (Waters), available to our research group allowed us to replace and update the previous TQD analyzer. Updates are valuable to show improvements (but also drawbacks) and novelties compared to "older" instruments. This was demonstrated in the second study by the improvement in sensitivity for all compounds, but also by the problematic which rose when analyzing smaller molecules, like amphetamine, which were studied in more detail. Besides these aspects, the scope of the method was enlarged, adding several opioids and ketamine. The latter is more and more detected in wastewaters suggesting an increase in consumption of this drug. Furthermore, a detailed study on reduction of sample load into the LC-MS/MS system, by injecting less volume and/or diluting the wastewater influents, in order to reduce matrix effect, was carried out evaluating the chromatographic performance and sensitivity. The developed method was applied to influent and effluent wastewaters collected over 14 consecutive sampling days, and it is currently being applied at our laboratory for the sub-ppb level quantification of these highly consumed IDs in wastewaters.

The results of these studies have been published in:

- *Journal of Chromatography A* 1216, **2009**, 3078 - 3089
- Analytical Bioanalytical Chemistry 406, **2014**, 4261- 4272

Chapter 2.2.1, scientific article 1

Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater

Lubertus Bijlsma, Juan V. Sancho, Elena Pitarch, Maria Ibáñez, Félix Hernández

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Simultaneous ultra-high-pressure liquid chromatography–tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater☆

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Abstract

An ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) method has been developed for the simultaneous quantification and confirmation of 11 basic/acidic illicit drugs and relevant metabolites in surface and urban wastewater at ng/L levels. The sample pre-treatment consisted of a solid-phase extraction using Oasis MCX cartridges. Analyte deuterated compounds were used as surrogate internal standards (except for norbenzoylecgonine and norcocaine) to compensate for possible errors resulting from matrix effects and those associated to the sample preparation procedure. After SPE enrichment, the selected drugs were separated within 6 min under UHPLC optimized conditions. To efficiently combine UHPLC with MS/MS, a fast-acquisition triple quadrupole mass analyzer (TQD from Waters) in positive-ion mode (ESI+) was used. The excellent selectivity and sensitivity of the TQD analyzer in selected reaction monitoring mode allowed quantification and reliable identification at the LOQ levels. Satisfactory recoveries (70–120%) and precision (RSD < 20%) were obtained for most compounds in different types of water samples, spiked at two concentration levels [limit of quantification (LOQ) and 10LOQ]. Thus, surface water was spiked at 30 ng/L and 300 ng/L (amphetamine and amphetamine-like stimulants), 10 ng/L and 100 ng/L (cocaine and its metabolites), 300 ng/L and 3000 ng/L (tetrahydrocannabinol-COOH). Recovery experiments in effluent and influent wastewater were performed at spiking levels of three and fifteen times higher than the levels spiked in surface water, respectively. The validated method was applied to urban wastewater samples (influent and effluent). The acquisition of three selected reaction monitoring transitions per analyte allowed positive findings to be confirmed by accomplishment of ion ratios between the quantification transition and two additional specific confirmation transitions. In general, drug consumption increased in the weekends and during an important musical event. The highest concentration levels were 27.5 µg/L and 10.5 µg/L, which corresponded to 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy) and to benzoylecgonine (a cocaine metabolite), respectively. The wastewater treatment plants showed good removal efficiency (>99%) for low levels of illicit drugs in water, but some difficulties were observed when high drug levels were present in wastewaters.

Keywords

Illicit drugs, ultra-high-Pressure liquid chromatography, tandem mass spectrometry, confrimation, matrix effects, triple quadrupole, urban wastewater

1. Introduction

The consumption of alcohol and illicit drugs during weekends and special events (e.g. concerts, festivals, local festivities) is significantly higher than on weekdays. Generally, drug consumption by individuals has been discovered by analyzing biological fluids (e.g., blood, serum, oral fluids, or urine) [1 - 4]. However, the estimation of drug consumption of an audience during these events is rather complicated and unreliable, due to the voluntary participation of the consumers in these specific analysis studies. An approach to obtain reliable data to monitor and estimate the consumption of illicit drugs of a population was proposed by Daughton in 2001 [5] and put into practise in 2005 by Zuccato et al. [6]. This approach, consisting of the determination of illicit drugs in urban wastewaters, got worldwide attention by the media and has been supported by various scientists [7 - 14], becoming a major issue nowadays. Information on accurate real-time data is of interest, both in environmental and social scientific studies. Results may also contribute to the evaluation of the removal efficiency of wastewater treatment plants (WWTPs) [11], to the understanding of the potential impact of these compounds on the aquatic ecosystem [15], and can help social scientists and authorities to combat drug abuse.

The analysis of illicit drugs in wastewater and surface water has been recently reviewed by Castiglioni et al. [16]. The authors pointed out that the principal difficulty deals with the low concentration levels of drugs in combination with the complexity of the matrix. Developed analytical methods are based on solid-phase extraction (SPE), for sample pre-treatment and pre-concentration, and the analytical technique of choice is liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS).

Drugs to be analyzed have been generally extracted using Oasis SPE polymer cartridges built of a balanced mixture of hydrophilic and lipophilic (HLB) monomers [8, 9, 11], or Oasis MCX, a strong cation-exchange mixed mode polymeric sorbent built upon HLB copolymers [6, 7]. The different properties (acid, neutral or basic) of the illicit drugs make the selection of SPE cartridges of great importance. Besides, the optimization of SPE process is required to avoid analyte losses, but SPE pre-

concentration, which is normally required to obtain the intended sensitivity, can also concentrate matrix interferences resulting in signal enhancement or suppression. In this way, the presence of matrix effects is found to be one of the main factors affecting the sensitivity of the established analytical method [17]. The addition of appropriate internal standards is one of the best approaches to compensate for matrix effects, especially when using analyte isotope labelled internal standard, as one expects that the internal standard is affected by matrix effects in the same way than the analyte [7, 12, 18 - 20]. When the internal standard is used as surrogate (i.e. added to the sample previously to sample treatment), it can also compensate for potential analytical errors associated to the sample manipulation.

In relation to LC–MS/MS methodology for the determination of illicit drugs, recent papers have reported methods using ultra-high-pressure liquid chromatography (UHPLC) coupled to MS/MS for ultra fast separations and reliable determination of these analytes [20 - 22]. Besides ultra fast separation, UHPLC allows better resolution, largely due to advancements in the particle size and bridging structure of the column packing. This advantage is important in terms of sample throughput and sensitivity improvement. Moreover, the use of MS/MS with triple quadrupole (QqQ) analyzers allows to minimize, or in some cases to eliminate, matrix interferences, improving selectivity and sensitivity due to the possibility of adequate precursor and product ion selection, leading to low chemical noise in the chromatograms. Thus, UHPLC–MS/MS QqQ in selected reaction monitoring (SRM) mode is considered nowadays as one of the most selective and sensitive techniques for the quantification and confirmation of organic contaminants in environmental samples and it is highly suitable for trace determination of drugs of abuse in water. However, the applications cited above do not take full advantage of UHPLC separations, because fast scanning triple quadrupole was not used. Therefore longer separations were needed.

The confirmation of the identity of compounds detected in samples, even at the limit of quantification (LOQ) level, is of great importance in order to avoid the reporting of false positives. Different approaches can be used for confirmation, most of them based on the collection of analyte mass information. One of the most frequently used

confirmation criteria [23] is based on the collection of identification points (IPs), which are earned depending on the mass analyzer used. Regarding MS/MS analysis with low resolution instruments, as QqQ, a minimum of two SRM transitions should be monitored for a safe positive finding, together with the measurement of the ion ratio between both recorded transitions. However, the quality of the transitions should be taken into account, as acquiring non-specific transitions might lead to report false positives or even false negatives, because of the non-compliance of the expected ion ratios [18, 24].

The aim of this work is to develop and validate a rapid and sensitive method for the simultaneous determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and in urban wastewater. The developed method, using Oasis MCX SPE cartridges for clean-up and pre-concentration, followed by UHPLC–MS/MS measurement, has been applied to the analysis of 24-h composite effluent and influent urban wastewaters samples from a WWTP of the Castellón province (Spain). Daily 24-h composite samples were collected along two complete weeks, one in June and another one in July 2008, during an important rock event. Special emphasis is placed on the reliable confirmation of illicit drugs detected in water, so that three SRM transitions have been acquired to simultaneously detect, quantify and confirm positive samples.

2. Experimental

2.1. Chemicals and materials

Illicit drugs and metabolites studied were the following: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA, or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, norcocaine, and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), a relevant metabolite of Δ^9-tetrahydrocannabinol (cannabis). These compounds were obtained from Sigma–Aldrich (Madrid, Spain), Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol, acetonitrile or as salt. Some features of the studied compounds such as chemical structure, mono-isotopic

molecular weight and CAS number are shown in **Fig. 1.** Standard stock solutions of each compound were prepared at 100 mg/L in methanol or acetonitrile. Intermediate solutions (10 mg/L) were prepared by diluting the stock solution ten times with methanol. Infusion solutions of individual standards were prepared at a concentration of 1.5 mg/L in methanol: water (50:50, v/v) just before infusion experiments. Mixed working solutions containing all analytes were prepared from intermediate solutions at different concentrations by appropriate dilution with water, and were used for preparation of the aqueous calibration standards and for spiking samples in the validation study.

Deuterated compounds were all purchased from Cerilliant as solutions in methanol or acetonitrile at a concentration of 100 mg/L and were used as surrogate internal standards for quantification: [$^2H_6$]amphetamine (amphetamine-d_6), [$^2H_5$]meth-amphetamine (methamphetamine-d_5), [$^2H_5$]3,4-methylenedioxy-amphetamine (MDA-d_5), [$^2H_5$]3,4-methylenedioxymethamphetamine (MDMA-d_5), [$^2H_5$]3,4-methylenedioxy-ethylamphetamine (MDEA-d_5), [$^2H_3$]benzoylecgonine (benzoylecgonine-d_3), [$^2H_3$]11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH-d_3), [$^2H_3$]cocaine (cocaine-d_3) and [$^2H_8$]cocaethylene (cocaethylene-d_8). A mixed surrogate standard working solution at 100 µg/L (except: THC-COOH-d_3 at 1 mg/L) was prepared in water.

All standard solutions were stored in amber glass bottles at −20 °C.

HPLC-grade methanol, ammonia solution (25%) and formic acid (98–100%) were acquired from Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA).

SPE cartridges, mixed reversed-phase/cation-exchange cartridges (Oasis-MCX; 6 mL, 150 mg) and SPE cartridges, built of a hydrophilic and a lipophilic monomer(Oasis-HLB; 6 mL, 200 mg) were purchased from Waters (Milford, MA, USA).

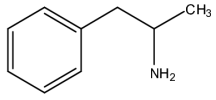
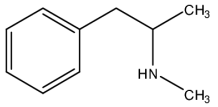
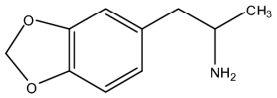
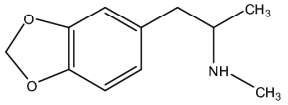
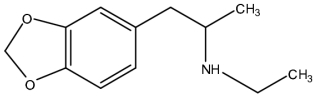
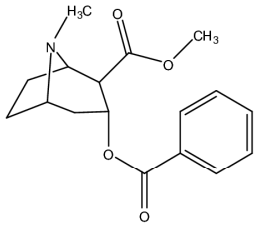
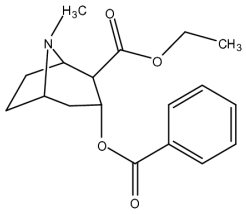
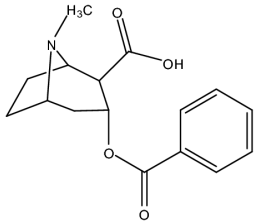
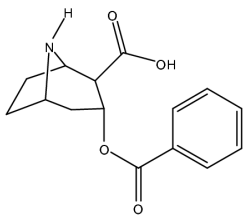
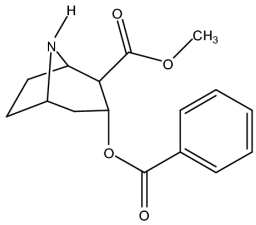
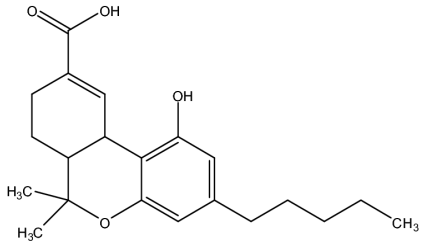
Chemical structure	Compound Mono-isotopic Molecular weight CAS number	Chemical structure	Compound Mono-isotopic Molecular weight CAS number
	Amphetamine 135.1 [300-62-9]		Methamphetamine 149.1 [537-46-2]
	MDA 179.1 [4764-17-4]		MDMA 193.1 [42542-10-9]
	MDEA 207.1 [82801-81-8]		Cocaine 303.1 [50-36-2]
	Cocaethylene 317.4 [529-38-4]		Benzoylecgonine 289.1 [519-09-5]
	Nor-benzoylecgonine 275.2 [60426-41-7]		Norcocaine 289.1 [61585-22-6]
			11-nor-9-carboxy-Δ^9 -tetrahydrocannabinol 344.5 [56354-06-4]

Figure 1. Chemical structure, mono-isotopic molecular weight, and CAS number of the selected illicit drugs.

2.2. Samples

A number of 28 wastewater samples were collected in polyethylene high density bottles and stored in the dark at −20 °C. The 24-h composite samples were taken from influent and effluent from a WWTP of the province of Castellón (Eastern Spain), during the third week of June 2008 (average week) and the third week of July 2008 (during an important music event). This WWTP is designed to treat wastewaters (urban or mixed urban and industrial) of a small population of approximately 32 000 inhabitants, with a mean flow rate of 8250 m^3/day. In this study, no surface water samples were taken. However, surface water was collected for validation for future studies, and it was taken in February 2008 from the Mijares River (Villarreal, Castellón).

2.3. Ultra-high-pressure liquid chromatography

A triple quadrupole mass spectrometer was interfaced to a Waters Acquity UPLC system (Waters). Chromatographic separation was carried out using an Acquity UPLC BEH C_{18} column (50 mm × 2.1 mm I.D., particle size 1.7 μm) (Waters). An optimized gradient was used at a constant flow rate of 0.3 mL/min using methanol (solvent A) and 5 mM ammonium acetate 0.1% formic acid (solvent B). The gradient elution was: 0 min, 10% A; 0–3 min linear from 10 to 90% A; 3–3.5 min, 90% A; 3.5–3.6 min linear from 90 to 10% A, return to initial conditions; 3.6–6 min 10% A, equilibration of the column.

2.4. Mass spectrometry

A TQD triple quadrupole mass spectrometer with an orthogonal electrospray ionization source Z-spray (Waters) was used. Cone gas as well as desolvation gas was dry nitrogen generated from pressurized air in a N_2 LC–MS (Claind, Teknokroma, Barcelona, Spain) nitrogen generator. The cone gas and the desolvation gas flows were optimized at approximately 60 L/h and 1100 L/h, respectively. For operation in the MS/MS mode, collision gas was Argon 99.995% (Praxair, Madrid, Spain) with a pressure of 2×10^{-3} mbar in the T-wave collision cell. Other parameters optimized were: capillary voltage, 3.5 kV in positive ionization mode; lens voltage 0.3 V; source temperature, 120 °C and desolvation temperature, 500 °C. Dwell times of 0.01 s/transition were selected.

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

2.5. Recommended procedure

Prior to solid-phase extraction all water samples were centrifuged for 5 min at 4500 rpm. 50 mL of surface water or effluent wastewater, or five times diluted influent wastewater, were spiked with a mixed surrogate labelled standard and the pH was adjusted to 2.0 with formic acid. The final concentration in sample for each surrogate labelled internal standard was 0.1 µg/L, except for THC-COOH (1 µg/L). SPE was performed using Oasis MCX cartridges that were conditioned by washing and rinsing with 6 mL of MeOH, 3 mL of Milli-Q water and 3 mL of acidified water (pH 2). Samples were percolated through the cartridges by gravity, and then cartridges were washed with 5 mL of 2% ammonia in water and vacuum dried for 15 min. Analytes were eluted using 8 mL of a 2% ammonia solution in MeOH.

The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen [20] and reconstructed in 1 mL of 10% methanol aqueous solution. The sample extract (20 µL) was injected directly into the UHPLC–MS/MS system without filtration (**Fig. 2**).

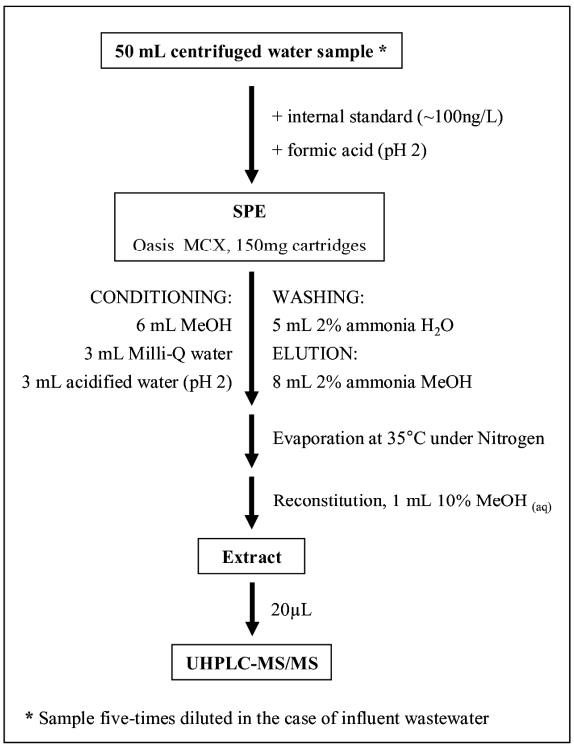


Figure 2. Diagram of the recommended procedure.

2.6. Quantification and method validation

Acquisition was performed in SRM mode, with the protonated molecular ion of each compound chosen as precursor ion. The most abundant product ion of each target analyte was typically used for quantification and two additional product ions were used for confirmation. LC retention time was also compared with that of the reference standards (within ±2.5%) to help to confirm the compounds detected in samples. Each compound was quantified using its corresponding labelled analyte as surrogate internal standard, except norcocaine, which was quantified using a deuterated analogue (cocaine-d_3), and norbenzoylecgonine, for which no adequate internal standard was found.

The linearity of the method was studied by analyzing standard solutions in triplicate at six concentrations, in the range from 2 µg/L to 70 µg/L for amphetamine and amphetamine-like stimulants; from 0.5 µg/L to 25 µg/L for cocaine and its metabolites; and from 20 µg/L to 600 µg/L for THC-COOH, depending on the sensitivity reached for each analyte. Satisfactory linearity was assumed when the correlation coefficient (*r*) was > 0.99, based on analyte/internal standard peak areas measurement.

The limit of quantification (LOQ) objective was taken as the lowest concentration level for which the method was fully validated using spiked samples with satisfactory recovery (between 70 and 120%) and precision (relative standard deviation (RSD) ≤ 20%). Confirmation by using three MS/MS transitions was also required at the LOQ level.

The limit of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated for a signal-to-noise of three from the quantitation SRM chromatograms of samples spiked at the lowest analyte concentration tested.

Accuracy (estimated by means of recovery experiments) and *precision* (expressed as repeatability in terms of RSD) were evaluated by analyzing water samples spiked at different concentration levels (LOQ and 10LOQ). In surface water, the LOQs objective were 30 ng/L for amphetamine and amphetamine-like stimulants, 10 ng/L for cocaine and its metabolites, and 300 ng/L for THC-COOH. In effluent and influent wastewater

the LOQs were around three and fifteen times higher, respectively. All recovery experiments were performed in quintuplicate for each type of water sample tested.

3. Results and discussion

3.1. MS/MS optimization

Full-scan mass spectra and MS/MS compound-dependent parameters (e.g. cone voltage, collision energy) were optimized by direct infusion of individual standard solutions at 1.5 mg/L in methanol/water (50:50, v/v) and at a flow rate of 10 µL/min, using the build-in syringe pump directly connected to the interface. All analytes were measured by electrospray ionization (ESI) operating in positive ionization mode, including THC-COOH. Optimum MS source and analyzer conditions for SRM determination of each target and internal standard compound are listed in **Table 1**.

TQD mass spectrometer is a fast-acquisition triple quadrupole mass analyzer that allows decreasing dwell times and ionization mode switching time, without apparent sensitivity losses. This gave us the possibility of acquiring up to three SRM transitions per compound at 10 ms dwell time. The most sensitive SRM transition was typically selected for quantification whereas two additional transitions were acquired to render a highly reliable confirmative method. Non-specific transitions, e.g. loss of water, were avoided as possible in order to minimize the risk of false positives [24]. Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (*Q*) and confirmation (*q*1 and *q*2) transitions and comparing them with mean *Q*/*q* value obtained from the calibration standards. The sample was considered positive when the experimental ion ratio fell within the tolerance range, in the line of the European Union Guidelines [23]. Retention time had also to fit with that of the reference standard (within ±2.5%) to be reported as positive.

Table 1. UHPLC-MS/MS parameters established for the SRM acquisition mode (quantification and confirmation transitions). For labelled internal standards, only typically the quantification related transition was acquired

Compounds	Rt (min)	Precursor ion (m/z) $[M + H]^+$	CV[a] (V)	CE[b] (eV)	Product ion[c] (m/z)	Q/q ratio (RSD %)
Amphetamine	1.47	136.2	25	20	91.1	
				10	119.1	0.56 (7)
				30	65.1	7.69 (9)
MDA	1.47	180.2	25	10	163.2	
				20	105.1	2.63 (7)
				20	133.1	3.00 (7)
MDEA	1.60	208.3	35	25	105.1	
				40	77.1	1.57 (7)
				25	135.1	1.82 (8)
MDMA	1.49	194.3	30	15	163.2	
				25	105.1	1.58 (4)
				40	77.1	2.83 (8)
Methamphetamine	1.50	150.3	35	20	91.1	
				10	119.1	2.65 (7)
				35	65.1	11.99 (9)
Cocaine	1.84	304.1	30	20	182.2	
				30	82.0	1.90 (9)
				50	77.0	2.93 (7)
Cocaethylene	2.05	318.3	45	20	196.2	
				30	82.0	9.74 (9)
				25	150.2	1.64 (8)
Benzoylecgonine	1.68	290.1	40	20	168.2	
				30	82.0	1.60 (8)
				30	105.0	0.81 (8)
Norbenzoylecgonine	1.76	276.2	45	15	154.1	
				20	136.1	1.61 (10)
				45	77.0	4.67 (14)
Norcocaine	1.94	290.1	30	15	136.1	
				25	168.2	0.85 (10)
				35	68.0	2.14 (10)
THC-COOH	3.68	345.3	40	15	193.2	
				25	299.3	0.46 (6)
				20	327.3	0.33 (10)
Amphetamine-d_6	1.45	142.2	25	20	93.1	
MDA-d_5	1.45	185.2	25	10	168.2	
MDEA-d_5	1.58	213.3	35	25	135.2	
MDMA-d_5	1.48	199.3	30	15	165.3	
Methamphetamine-d_5	1.49	155.3	35	20	92.3	
Cocaine-d_3	1.83	307.1	30	20	185.3	
Cocaethylene-d_8	2.05	326.3	45	20	204.3	
Benzoylecgonine-d_3	1.69	293.1	40	20	171.2	
THC-COOH-d_3	3.67	348.3	40	15	330.3	

[a]CV, cone voltage; [b]CE, collision energy; [c]Top, product ion used for quantification; Below, the two product ions used for confirmation

As a consequence of the higher flow rate of the mobile phase, an elevated desolvation temperature was necessary. By increasing the desolvation temperature and the desolvation gas flow rate, more reproducible data were acquired, which finally lead to a more robust method. In order to improve desolvation efficiency, analyte ionization and reproducibility, the following parameters were optimized and set up as follows: source temperature, 120 °C; desolvation temperature, 500 °C; cone gas flow rate, 60 L/h; desolvation gas flow rate, 1100 L/h.

Determination of THC-COOH has been reported both in negative-ion mode [atmospheric pressure chemical ionization (APCI) or ESI] [7, 12, 25], and in positive-ion mode [21]. In principle, more abundant ionization would be obtained in negative mode, due to the expected higher trend towards the ionization of the acidic group. However, in our work THC-COOH presented more abundant ionization in ESI positive-ion mode, which would indicate better ionization towards basic groups. In addition to the higher sensitivity, ionization in positive-ion mode was also of our interest in order to get a simultaneous determination method for all analytes selected using the same LC separation. Special effort was done to optimize sample preparation and LC separation to incorporate THC-COOH in our method, but we still observed some difficulties related to the confirmatory capability and sensitivity for this analyte. According to Postigo et al. [12] a single relevant indicator of cannabinoid consumption cannot be pointed out yet. Nevertheless, we expected to have a rough estimation of the consumption of cannabis by determining THC-COOH, which has been used as an indicator of cannabis usage by other authors as well [7, 21].

3.2. UHPLC optimization

Chromatographic separation might not be a crucial issue when using MS/MS for detection, because the probability of finding two compounds with the same retention time and the same SRM transitions is fairly low. However, in LC–MS/MS, an efficient LC separation is important to avoid or minimize matrix effects. In addition, the selection of the mobile phase composition can be relevant to enhance the detector response [17].

In this paper, different mobile phase compositions – variations of buffer (ammonium acetate and ammonium formate), pH (addition of formic acid) and organic solvents (acetonitrile, methanol) – were tested. The effects of pH and mobile phase ionic strength in the peak shapes, resolution and efficiencies were evaluated by varying the buffer concentration. An optimum mobile phase consisting of ammonium acetate (5 mM) with 0.1% formic acid (pH 2) and methanol was selected. This mobile phase showed satisfactory results for the drugs selected, and also for THC-COOH, the compound with lower sensitivity. Applying the UHPLC recommended gradient shown in Section 2.3, the selected drugs were separated within 4 min.

As it can be seen in **Table 1** and **Fig. 3**, benzoylecgonine and norcocaine presented a common transition (290.1 → 168.2). In this particular case, chromatographic separation becomes an important issue and co-elution needs to be avoided. In our case, this transition was used for quantification of benzoylecgonine being necessary a complete resolution with norcocaine to obtain reliable data. From **Fig. 3**, the baseline peak-to-peak resolution (*R*) for benzoylecgonine and norcocaine was calculated to be 1.8. A value which illustrates complete resolution of both peaks [26].

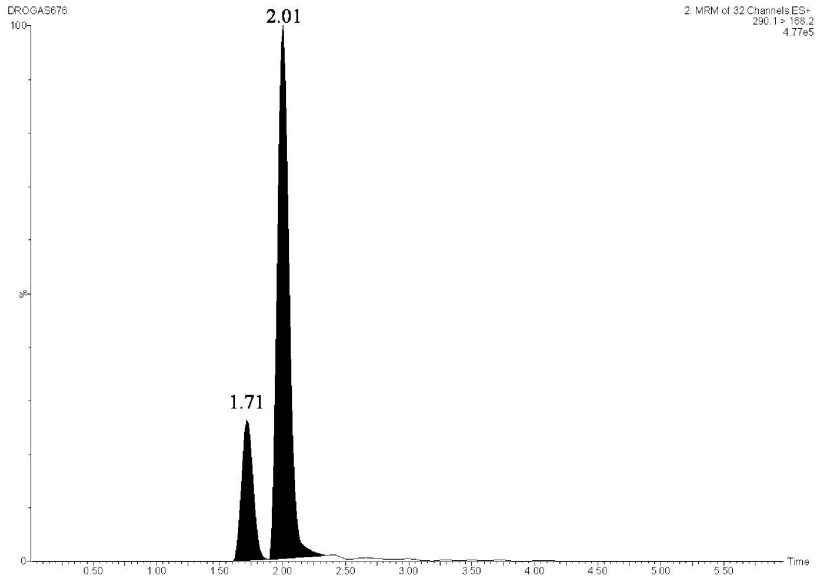


Figure 3. UHPLC-MS/MS chromatogram (SRM transition 290.1 → 168.2) from a mixed reference standard of benzoylecgonine (1.6 µg/L, Rt = 1.71 min) and norcocaine (1.8 µg/L, Rt = 2.01 min).

3.3. Sample preparation and matrix effects

During the optimization of the SPE process, two different cartridges were evaluated: Oasis HLB and Oasis MCX. Their extraction efficiencies were estimated from the recovery percentage obtained for each target compound when loading a small sample volume (50 mL) of Milli-Q water at a low sample loading flow rate (gravity) (triplicate analysis). 50 mL Milli-Q water sample was spiked with a mixed standard solution at individual concentrations of 2 µg/L. All selected analytes showed satisfactory absolute recoveries (80–120%) for both cartridges. Oasis MCX were selected for further optimization with surface water, because the mixed mode material allows improved selectivity towards basic analytes due to pH and polarity changes during loading, washing and elution steps. The MCX cartridges were washed with aqueous basic solution (2% ammonium in water) instead of organic (methanol), to remove interferences such as inorganic salts, which resulted in lower matrix effect. We observed that the washing had no negative effect on the recoveries or on the sensitivity of the target analytes. Finally, analytes were eluted from the cartridges using a 2% ammonium solution in MeOH.

Filtration of the extract is of importance in order to maintain maximum life-time of the sub-2-µm analytical column. Consequently we tested analyte losses due to the filtering of extracts before injecting into the UHPLC–MS/MS system. In this work, 0.2 µm filters (13 mm) of different materials (polypropylene and PTFE) were tested, but the results were not satisfactory, showing recoveries lower than 70% for cocaine, cocaethylene, norcocaine and THC-COOH. At the end, we decided not to filter the final extracts before injection into the UHPLC system, until an adequate material is found.

Absolute recoveries, to estimate the extraction efficiency of the MCX cartridges, are shown in **Fig. 4A**. In the case of surface water, recoveries were slightly lower (65–94%) compared to Milli-Q water and for three compounds (amphetamine, methamphetamine and THC-COOH) values below 70% were obtained. We assumed that absolute recoveries of selected analytes in effluent and influent wastewaters would possibly be worse, as these sample matrices are more complex and problematic from an analytical point of view. Therefore, the internal standards were added to the

samples as surrogates, just before the SPE process, to compensate for potential losses of compounds along sample treatment.

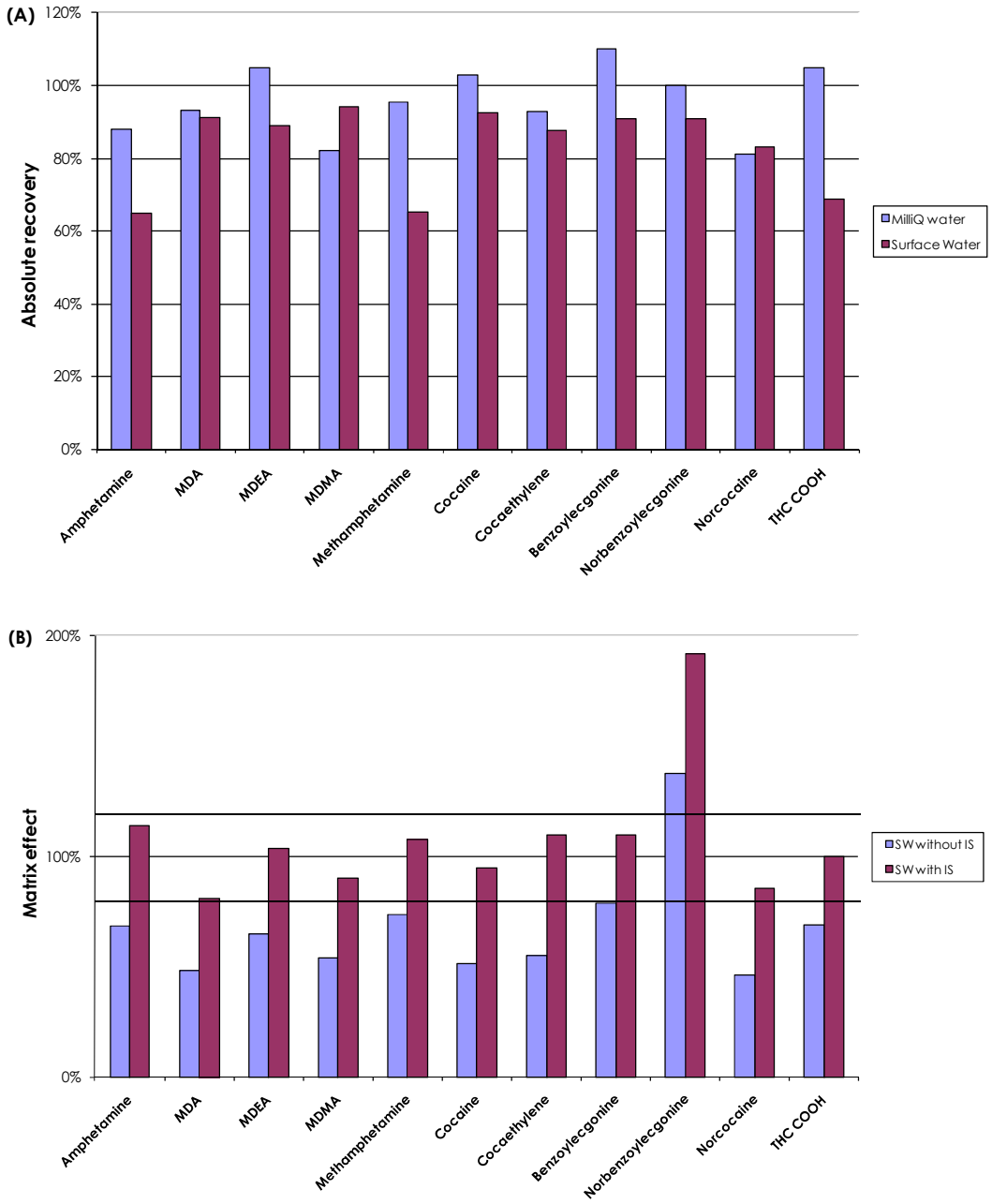


Figure 4. (A) Absolute recoveries of all selected analytes in Milli-Q and surface water using MCX cartridges. (B) Matrix effects in surface water for all selected analytes with and without correction of internal standards.

Matrix effects can lead to a suppression or enhancement of the analyte response due to co-eluting matrix constituents. Several approaches are typically applied to deal with matrix effects in quantitative analysis: improvement of the sample pre-treatment and/or the chromatographic separation, matrix-matched standards calibration, sample dilution or the use of stable-isotopically labelled internal standards, the latest being widely accepted to be the most satisfactory approach [17, 18]. Preliminary experiments on surface water were performed by spiking SPE extracts in order to avoid the influence of potential losses in the SPE process. Therefore, SPE blank surface water extracts were spiked with the selected drugs (native and labelled) and matrix effects were evaluated for each compound calculating the absolute (without internal standard) and relative (with internal standard) responses in comparison to those of reference standards in solvent. Matrix ionization suppression was observed for all compounds, except for norbenzoylecgonine, which showed ionization enhancement (~200%) (**Fig. 4B**). As the deuterated analyte was not available for this compound, benzoylecgonine-d_3 was tested as analogue IS to compensate for norbenzoylecgonine matrix effects, but the results were not satisfactory in none of the samples tested. For the rest of compounds, the use of labelled IS corrected the matrix effects, as expected.

An estimation of matrix effects in wastewater samples could be made throughout the signal variations observed in the deuterated IS, which were added to all type of samples as well as to standards in solvent. As expected, matrix effects seemed to be more important in wastewater (mainly in the influent) and notable ionization suppression was observed for all analytes, norbenzoylecgonine included.

In our work, to compensate for potential errors associated to both sample preparation and matrix effects, each compound was quantified using its corresponding deuterated standard added as surrogate internal standard. In the case of norcocaine and norbenzoylecgonine, their labelled analogues were not commercially available; so norcocaine was quantified using cocaine-d_3, for norbenzoylecgonine no labelled standards were found suitable and it was quantified without using a surrogate internal standard. Besides, influent wastewater needed to be at least five times diluted before

the SPE process to handle with matrix effect. Otherwise, the ionization suppression was so high that it could not be properly compensated even using deuterated standards.

Before SPE, water samples were centrifuged for 5 min at 4500 rpm to avoid compound losses that could take place if filtration was used instead. After centrifugation, the surrogate/internal standards were added, assuring in this way the correct quantification of analytes in real water samples.

3.4. Method validation

Prior to its application, the overall analytical procedure was satisfactorily validated for surface water (**Table 2**), effluent wastewater (**Table 3**) and influent wastewater (**Table 4**), considering the following parameters: linearity, precision and accuracy, LOQs, LODs, and *Q*/*q* ratios used for confirmation. These tables show relative recovery as surrogate/IS were used in validation experiments.

Table 2. Method validation in surface water (n = 5)

Compound	Recovery (RSD)		LOD (ng/L)	Q/q_1 ratio deviation (%)[b]	Q/q_2 ratio deviation (%)[b]
	LOQ[a]	10LOQ			
Amphetamine	102 (5)	95 (6)	2	4 (from -10 to +7)	6 (from +1 to +12)
MDA	106 (7)	96 (6)	17	1 (from -10 to +10)	8 (from -3 to +25)
MDMA	92 (13)	97 (9)	4	1 (from -8 to +6)	10 (from +3 to +17)
MDEA	94 (4)	96 (3)	0.5	3 (from -6 to +16)	4 (from -12 to +15)
Methamphetamine	90 (7)	98 (6)	0.6	6 (from -17to +19)	6 (from -25 to +33)
Cocaine	90 (15)	76 (9)	0.8	6 (from -14 to +17)	0 (from -19 to +20)
Cocaethylene	70 (9)	82 (3)	0.3	11 (from +3 to +22)	4 (from -18 to +19)
Benzoylecgonine	103 (12)	84 (8)	0.05	5 (from -4 to +13)	3 (from -12 to +14)
Norbenzoylecgonine	92 (6)	107 (5)	2	5 (from -13 to +6)	4 (from -3 to +15)
Norcocaine	73 (11)	61 (7)	2	7 (from -20 to +0)	4 (from -13 to +17)
THC COOH	91 (14)	120 (11)	30	3 (from -19 to +11)	2 (from -14 to +12)

[a] LOQ objective was 0.03 µg/L for amphetamine and amphetamine-like stimulants; 0.01 µg/L for cocaine and its metabolites; 0.3 µg/L for THC-COOH

[b] Average deviation and range (in %) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated (n=5) in relation to those calculated from reference standard solutions in solvent (see Table 1)

Table 3. Method validation in effluent wastewater (n = 5)

Compound	Recovery (RSD)		LOD (ng/L)	Q/q_1 ratio deviation (%)[b]	Q/q_2 ratio deviation (%)[b]
	LOQ[a]	10LOQ			
Amphetamine	102 (5)	103 (9)	40	8 (from +5 to +17)	14 (from +2 to +26)
MDA	88 (5)	77 (9)	88	1 (from -9 to +10)	3 (from -6 to +10)
MDMA	84 (25)	100 (13)	9	6 (from +2 to +14)	4 (from -5 to +18)
MDEA	103 (3)	105 (2)	9	5 (from +1 to +13)	7 (from +1 to +23)
Methamphetamine	94 (17)	84 (16)	1	3 (from -25 to +24)	13 (from +7 to +27)
Cocaine	109 (4)	125 (1)	2	4 (from -8 to +16)	9 (from +4 to +14)
Cocaethylene	78 (11)	97 (8)	1	1 (from -22 to +20)	4 (from -16 to +14)
Benzoylecgonine	120 (7)	114 (14)	0.3	5 (from -16 to +8)	1 (from -6 to +10)
Norbenzoylecgonine	80 (9)	63 (3)	0.2	7 (from -20 to -1)	9 (from -27 to 0)
Norcocaine	76 (5)	70 (9)	2	3 (from -11 to +5)	9 (from -14 to +5)
THC COOH	48 (8)	118 (11)	500	1 (from -17 to +17)	13 (from -20 to +1)

[a] LOQ objective was 0.1 µg/L for amphetamine and amphetamine-like stimulants; 0.03 µg/L for cocaine and its metabolites; 0.8 µg/L for THC-COOH

[b] Average deviation and range (in %) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated (n=5) in relation to those calculated from reference standard solutions in solvent (see Table 1)

Table 4. Method validation in influent wastewater (n = 5)

Compound	Recovery (RSD)		LOD (ng/L)	Q/q_1 ratio deviation (%)[b]	Q/q_2 ratio deviation (%)[b]
	LOQ[a]	10LOQ			
Amphetamine	113 (8)	108 (6)	54	3 (from -17 to +24)	6 (from -22 to +15)
MDA	90 (7)	81 (9)	91	5 (from -15 to +12)	6 (from -24 to +21)
MDMA	73 (5)	75 (8)	18	5 (from -15 to +13)	3 (from -8 to +10)
MDEA	75 (18)	71 (2)	40	2 (from -5 to +16)	4 (from -19 to +18)
Methamphetamine	116 (12)	119 (6)	7	3 (from -19 to +12)	2 (from -24 to +19)
Cocaine	87 (10)	112 (3)	3	0 (from -5 to +15)	0 (from -7 to +7)
Cocaethylene	85 (5)	95 (3)	2	4 (from -11 to +3)	6 (from -12 to +2)
Benzoylecgonine	c	c	d	1 (from -6 to +12)	4 (from -5 to +14)
Norbenzoylecgonine	50 (15)	57 (10)	1	1 (from -17 to +10)	11 (from +1 to +25)
Norcocaine	73 (8)	83 (5)	5	5 (from -2 to +12)	2 (from -7 to +15)
THC COOH	72 (9)	120 (6)	2500	2 (from -8 to +11)	1 (from +1 to +23)

[a] LOQ objective was 0.5 µg/L for amphetamine and amphetamine-like stimulants; 0.15 µg/L for cocaine and its metabolites; 4.0 µg/L for THC-COOH

[b] Average deviation and range (in %) of the experimental Q/q ratios obtained from sample extracts spiked at the lowest level validated (n=5) in relation to those calculated from reference standard solutions in solvent (see Table 1)

[c] Not calculated due to the high concentration found in the "blank" sample

[d] Not estimated, as validation at the LOQ level was unfeasible due to high levels found in the "blank" sample.

Linearity was studied for all selected compounds. A six-point calibration curve, in the range from 2 µg/L to 70 µg/L for amphetamine and amphetamine-like stimulants; 0.5 µg/L to 25 µg/L for cocaine and its metabolites; 20 µg/L to 600 µg/L for THC-COOH, was generated by injecting mixed standard solutions with a fixed amount of mixed surrogate internal standard solution. Correlation coefficients obtained were, with few exceptions, greater than 0.99.

Precision and accuracy were evaluated by spiking "blank" water samples at two concentration levels (LOQ and 10LOQ), and analyzing them in quintuplicate. It was difficult to obtain genuine blank samples for wastewater, specially for influent. "Blank" samples were collected from the WWTP on Tuesday when lower concentration of drugs were expected in comparison to weekends. These "blank" samples were previously analyzed and positive findings were subtracted from the spiked samples. In surface water, the LOQs objective were fixed at 30 ng/L for amphetamine and amphetamine-like stimulants, at 10 ng/L for cocaine and its metabolites, and at 300 ng/L for THC-COOH. In effluent and influent wastewater the LOQs were around three and fifteen times higher, respectively. The LOQ objective was established depending on the sensitivity of the method for each analyte and on the type of water. Besides, all three SRM transitions could be acquired at these low levels for all analytes, making the reporting data highly confident as regards the identity of the compound detected. The method was found highly specific as no relevant interferences were observed in the blanks at the analytes retention times for each transition.

In general, recoveries (between 70 and 120%) and precision (RSD < 20%) were satisfactory for most compounds at both fortification levels. **Table 3** shows data for effluent wastewater. As can be seen THC-COOH showed poor recovery (48%), but with satisfactory RSD (8%). At the 10LOQ level, cocaine showed a relatively high recovery (125%), but with good precision (1%). In influent wastewater (**Table 4**), benzoylecgonine precision and accuracy could not be calculated due to the high concentration found in the blank (around 2.5 µg/L). In both, effluent and influent wastewater, norbenzoylecgonine showed lower relative recoveries (in the range of 50–80%), but satisfactory RSD (<15%). The reason for the lower relative recoveries of

norbenzoylecgonine was probably owing to the absence of an adequate internal standard.

As can be seen in **Table 2**, **Table 3** and **Table 4**, the worst LODs were obtained for THC-COOH as a consequence of the poor sensitivity for this compound. With the exception of this metabolite, LODs in surface water varied between 0.05 ng/L and 17 ng/L, while in effluent wastewater increased up to the range 0.2–88 ng/L. In influent wastewater, LODs were in the range 1–91 ng/L. Regarding LOQs, we did not estimate them from a statistic point of view (e.g. from a signal-to-noise of 10). Instead, we have used a LOQ objective, which was established as the lowest level in sample for which the method was fully validated in terms of accuracy and precision. This criterion is normally applied in the field of pesticide residue analysis, accordingly to SANCO guidelines [27 and 28] and it leads to realistic LOQ values, normally higher than those estimated from statistical criteria. Besides, all compounds could be confirmed at the LOQ level by acquiring two additional transitions, in accordance with the two corresponding Q/q ratios.

Average intensity Q/q_1 and Q/q_2 ratios calculated from reference standards in solvent (see **Table 1**) were compared to those experimentally obtained from sample extracts spiked at the lowest level validated (i.e., the worst-case scenario). The aim was to test the robustness of these values and to check for potential matrix interferences that could affect the Q/q ratios and consequently, the confirmation process. As **Table 2**, **Table 3** and **Table 4** show, average Q/q_1 and Q/q_2 deviations were always below 20% (the maximum permitted tolerance varies between 20 and 50%, depending on the relative ion intensities (% of base peak)) [23].

3.5. Application to real samples

A number of 14 influent and 14 effluent 24-h composite wastewater samples were analyzed by the developed UHPLC–MS/MS method. 24-h composite water samples of Thursday and Sunday (of both June and July) have been selected as illustrative examples for a weekday and weekend, respectively, and real-time data obtained are summarized in **Table 5**.

Table 5. Real-time concentrations of illicit drugs in influent and effluent wastewater of a WWTP placed in Castellon province, during weekdays (1) and weekends (2) of June and July, 2008

Analyte	influent wastewater (µg/L)				effluent wastewater (µg/L)			
	June		July		June		July	
	weekday	weekend	weekday	weekend	weekday	weekend	weekday	weekend
Amphetamine	d* 1 (< 0.5)	d 2 (< 0.5)	- 1 (0.62)	1.40 3 (0.61-1.40)	-	-	-	0.21 2 (0.11-0.21)
MDA	-	-	d 2 (< 0.5-0.79)	1.69 3 (0.92-1.69)	-	-	- 1 (0.41)	0.68 3 (0.50-0.68)
MDEA	-	-	-	d 2 (< 0.5)	-	-	-	- 1 (< 0.1)
MDMA	-	d 2 (< 0.5)	3.26 4 (< 0.5-15.9)	27.5 3 (18.3-27.5)	d 2 (< 0.1)	d 3 (< 0.1)	0.71 4 (0.20-6.23)	21.2 3 (14.9-21.2)
Methamphetamine	-	-	-	d 2 (< 0.5)	d 1 (< 0.1)	d 1 (< 0.1)	-	d 1 (< 0.1)
Cocaine	0.82 3 (0.41-0.82)	0.50 3 (0.50-0.70)	0.55 4 (0.37-1.24)	0.56 3 (0.47-0.72)	d 3 (< 0.03)	d 3 (< 0.03-0.04)	d 4 (< 0.03-0.15)	0.54 3 (0.05-0.56)
Cocaethylene	d 3 (< 0.15)	d 3 (< 0.15)	d 3 (< 0.15-0.19)	0.15 3 (< 0.15-0.15)	-	-	d 2 (<0.03)	0.08 3 (< 0.03-0.08)
Benzoylecgonine	2.18 4 (1.48-2.18)	2.81 3 (2.15-2.81)	4.14 4 (1.65-7.21)	10.5 3 (5.67-10.5)	d 3 (< 0.03)	d 3 (< 0.03)	0.06 4 (< 0.03-1.45)	6.79 3 (0.76-6.79)
Norbenzoylecgonine	d 4 (< 0.15)	0.15 3 (< 0.15-0.15)	d 2 (< 015-0.31)	0.43 3 (0.20-0.43)	d 1 (< 0.03)	d 3 (< 0.03)	0.03 2 (0.03-0.11)	0.15 3 (0.12-0.17)
Norcocaine	-	-	-	-	d 4 (< 0.03)	d 3 (< 0.03)	d 3 (<0.03)	d 3 (0.03)
THC COOH	-	d 1 (< 4.0)	d 1 (< 4.0)	d 2 (< 4.0)	-	-	-	d 1 (< 0.8)

d: detected at concentration level ≥ LOD and ≤ LOQ

(1) Representative 24-h composite influent and effluent wastewater samples of a weekday (collected on Thursday 19th of June 2008, and on Thursday 17th of July 2008)

(2) Representative 24-h composite influent and effluent wastewater samples of a weekend (collected on Sunday 22nd of June 2008 and on Sunday 20th of July 2008)

* Top: individual concentration value found in the sample

Bottom: number of days when target analytes were positively identified, and concentration range (in brackets). A week is considered from Tuesday to Friday (4 days), a weekend is considered from Saturday to Monday (3 days)

In every sequence of analysis, water samples were injected in duplicate between two calibration curves. Two quality control samples (QCs), i.e. a blank water sample (previously analyzed) spiked at LOQ and 10LOQ levels, were also analyzed. QC recoveries were considered satisfactory when they were in the range of 70–120% for each analyte.

In general, concentrations of illicit drugs on weekdays were lower than during weekends. The consumption during the special musical event (July 2008) was considerably higher. THC-COOH showed a similar trend, although samples were not quantified, as all were below the LOQ objective of the method. In influent wastewater, cocaine and its main metabolite benzoylecgonine were the most abundant in the samples collected in June, with concentrations around 0.8 µg/L and 0.5 µg/L (cocaine) and 2.2 µg/L and 2.8 µg/L (benzoylecgonine), on weekdays and weekends, respectively. Benzoylecgonine was the most abundant cocaine metabolite in wastewater in all the samples analyzed and its concentration levels notably increased in July. This metabolite reached concentrations as high as 4.14 µg/L and 10.5 µg/L in influent wastewater on weekdays and weekends, respectively. It can be pointed out the impressive increase of amphetamine and amphetamine-like stimulants concentrations in July (especially MDMA with 3.26 µg/L and 27.5 µg/L in samples collected on Thursday and Sunday, respectively) during a special music event, which indicates that these drugs were mainly used for this special occasion. On the other hand, cocaine showed more constant concentration during the weeks analyzed, similar to the values found by Huerta-Fontela et al. [11], indicating a different pattern of use. Nevertheless, concentrations of benzoylecgonine, the main metabolite of cocaine, also increased significantly in July.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q_1 and q_2) transitions and comparing them with mean Q/q_1 and Q/q_2 values obtained from the calibration standards in the same sequence of samples. To consider a finding as positive, the experimental Q/q_i ratios should fit with those of reference standards with a maximum deviation ranging from 20 to 50% depending on the relative ion intensities. In our work, all findings were

confirmed by the two Q/q_1 and Q/q_2 ratios when analyte concentration was above the LOQ of the method. **Fig. 5** shows a positive finding of benzoylecgonine in influent and effluent wastewater. Samples, where analytes were detected at very low concentration levels (below the LOQ), were also considered as positive findings when all the three transitions acquired (with few exceptions) were observed and at least one Q/q ratio was within tolerance limits. Due to the higher chemical background in the chromatograms and to the low peak intensity as a consequence of the low analyte concentration, compliance of Q/q ratio of both confirmatory transitions was not always possible.

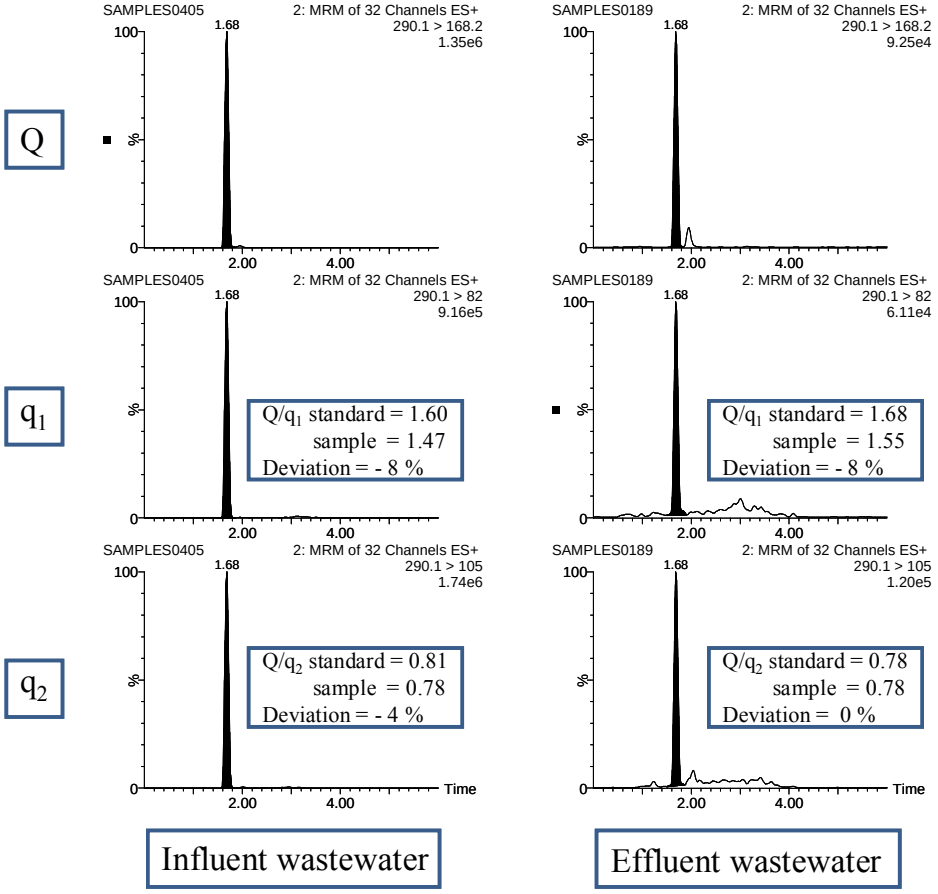


Figure 5. UHPLC-MS/MS chromatograms corresponding to the positive findings of benzoylecgonine in influent (4140 ng/L) and effluent (60 ng/L) 24-h composite wastewater sample from a weekday of July 2008. (Q) quantification transition; (q_1) and (q_2) confirmation transition.

The WWTP, designed to treat wastewaters (urban or mixed urban and industrial) of a small population, seemed to have good removal efficiency (>99%) for all compounds (see **Table 5**), but some difficulties were observed when high drug levels were present in wastewater samples (July). On weekdays, removal efficiency for MDMA and benzoylecgonine was around 80 and 95%, respectively. However, in samples collected on weekends, when the highest concentrations were reached, efficiency decreased to 25% (MDMA) and to 35% (benzoylecgonine). For other compounds detected, efficiencies were around 85% (amphetamine), 60% (MDA) and 65% (norbenzoylecgonine). Remarkable was the low removal efficiency observed for cocaine in the weekend sample of July (0.56 µg/L influent, 0.54 µg/L effluent), although this fact is in contradiction with the other samples analyzed.

Illustrative chromatograms (quantitative transition) are shown in **Fig. 6** for a weekend effluent sample (Sunday), which contained almost all compounds investigated in this work. The excellent sensitivity of the method and its potential to detect low concentration levels of analytes are proved. Data concentrations in this sample are found in **Table 5**, where it can be seen that methamphetamine, norcocaine and THC-COOH are reported as detected (i.e. concentration below the LOQ). Having a look to the corresponding chromatograms, one realizes that satisfactory peaks were obtained for these analytes when acquiring the quantification transition. The reason for not performing quantification in these samples is because the method was not validated at these low levels by acquiring both the quantification and confirmation transitions. Therefore, although quantitative data could have been reported at lower levels, we assumed a conservative criterion and reported these analytes as detected (concentrations above the LOD of the method, but below the LOQ). The high number of analytes detected in this effluent weekend sample leads to the conclusion that the efficiency removal of the WWTP was poor along that day (20 July 2008), where high concentrations of illicit drugs were present in the influent sample. This fact might have possible consequence for aquatic ecosystems [15].

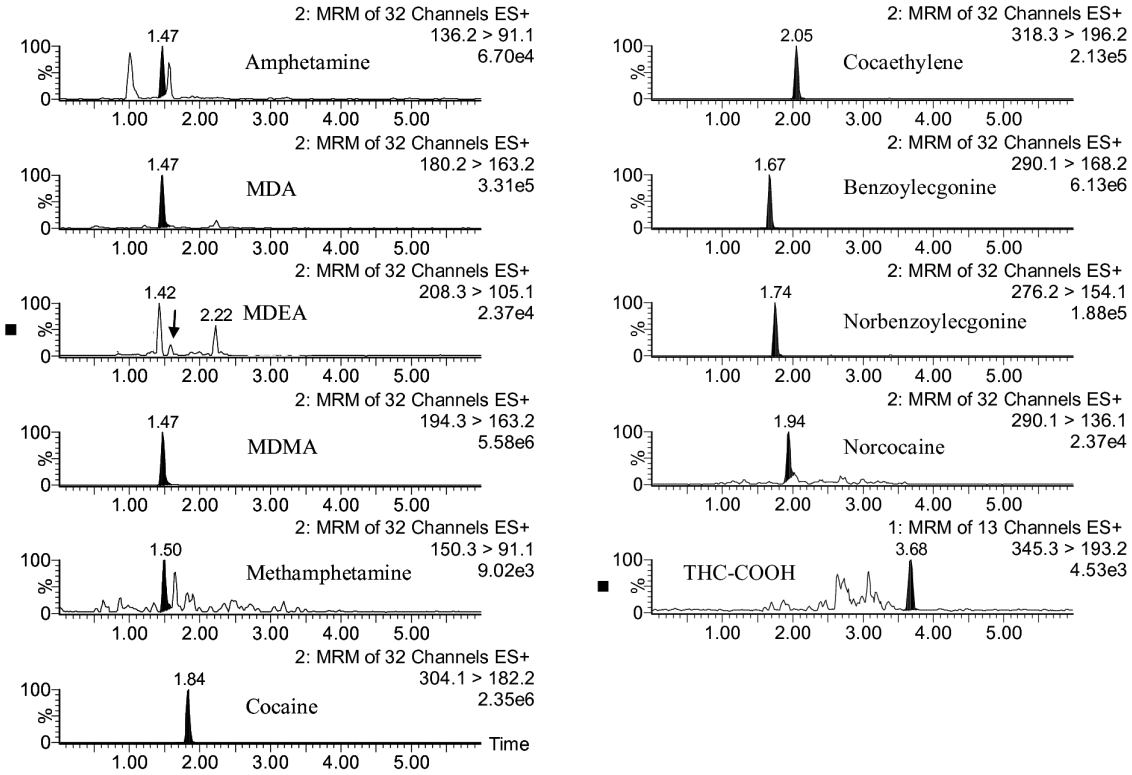


Figure 6. UHPLC-MS/MS chromatograms (quantification transition) for an effluent wastewater sample collected on a weekend (20 July 2008). See Table 5 for concentration data.

4. Conclusions

An analytical method based on UHPLC–MS/MS has been developed for the simultaneous quantification and confirmation of basic/acidic illicit drugs and relevant metabolites in surface and urban wastewater at the ng/L levels. Potential analytical errors associated to sample preparation and those resulting from matrix effects were compensated by using the analyte deuterated compound as surrogate internal standard. The overall analytical procedure, based on an off-line SPE step using Oasis MCX cartridges prior to the determination by UHPLC–MS/MS using a triple quadrupole analyzer, has been fully validated at the LOQ and 10LOQ level, obtaining satisfactory accuracy and precision. Confirmation of the analyte identity was guaranteed by acquiring 3 SRM transitions and the accomplishment of the ion ratios deviations for all analyte/matrix combinations, even at the LOQ level. The LOQ objective was established as a function of the sensitivity for each analyte, and in some cases was as low as 10 ng/L.

The application of this method to influent and effluent urban wastewater samples showed an increase of drug consumption during weekends and special events (e.g. festivals). The higher concentration levels reported corresponded to MDMA (ecstasy) and to the cocaine-metabolite benzoylecgonine. All positive findings were confirmed by accomplishment of ion ratios between the quantification transition (Q) and two specific additional confirmation transitions (*q*). The removal efficiency of the WWTP was satisfactory for low levels of illicit drugs in influent wastewater.

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References

[1] K.A. Mortier, K.E. Maudens, W.E. Lambert, K.M. Clauwaert, J.F. Van Bocxlaer, D.L. Deforce, C.H. Van Peteghem, A.P. De Leenheer, J. Chromatogr. B, 779 (2002), p. 321

[2] K. Nakashima, J. Health Sci., 51 (2005), p. 272

[3] H.H. Mauer, Anal. Bioanal. Chem., 388 (2007), p. 1315

[4] T.M. Pizzolato, M.J. Lopez de Alda, D. Barceló, Trends Anal. Chem., 26 (2007), p. 609

[5] C.G. Daughton, Am. Chem. Soc. Symp. Ser., 791 (2001), p. 348

[6] E. Zuccato, C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea, R. Fanelli, Environ. Health, 4 (14) (2005) http://www.ehjournal.net/content/4/1/14

[7] S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati, Anal. Chem., 78 (2006), p. 8421

[8] D. Hummel, D. Löffler, G. Fink, T.A. Ternes, Environ. Sci. Technol., 40 (2006), p. 7321

[9] A. Kaleta, M. Fredig, W. Buchberger, J. Sep. Sci., 29 (2006), p. 1662

[10] J. Bones, K.V. Thomas, B. Paull, J. Environ. Monit., 9 (2007), p. 701

[11] M. Huerta-Fontela, M.T. Galceran, J. Martin-Alonso, F. Ventura, Sci. Tot. Environ., 397 (2008), p. 31

[12] C. Postigo, M.J. Lopez de Alda, D. Barceló, Anal. Chem., 80 (2008), p. 3123

[13] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Anal. Bioanal. Chem., 391 (2008), p. 1293

[14] A. Gheorge, A. van Nuijs, B. Pecceu, L. Bervoets, P.G. Jorens, R. Blust, H. Neels, A. Covaci, Anal. Bioanal. Chem., 391 (2008), p. 1309

[15] E. Zuccato, S. Castiglioni, R. Bagnati, C. Chiabrando, P. Grassi, R. Fanelli, Water Res., 42 (2008), p. 961

[16] S. Castiglioni, E. Zuccato, C. Chiabrando, R. Fanelli, R. Bagnati, Mass Spectrom. Rev., 27 (2008), p. 378

[17] W.M.A. Niessen, P. Manini, R. Andreoli, Mass Spectrom. Rev., 25 (2006), p. 881

[18] F. Hernández, J.V. Sancho, O.J. Pozo, Anal. Bioanal. Chem., 382 (2005), p. 934

[19] B.J. Vanderford, S.A. Snyder, Environ. Sci. Technol., 40 (2006), p. 7312

[20] M. Huerta-Fontela, M.T. Galceran, F. Ventura, Anal. Chem., 79 (2007), p. 3821

[21] M.R. Boleda, M.T. Galceran, F. Ventura, J. Chromatogr. A, 1175 (2007), p. 38

[22] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, J. Chromatogr. A, 1161 (2007), p. 132

[23] European Commission Decision 2002/657/EC (17 August 2002), Off. J. Eur. Commun., 221 (2002), p. 8

[24] O.J. Pozo, J.V. Sancho, M. Ibañez, F. Hernandez, W.M.A. Niessen, Trends Anal. Chem., 25 (2006), p. 1030

[25] W. Weinmann, M. Goerner, S. Vogt, R. Goerke, S. Pollak, Foren. Sci. Int., 121 (2001), p. 103

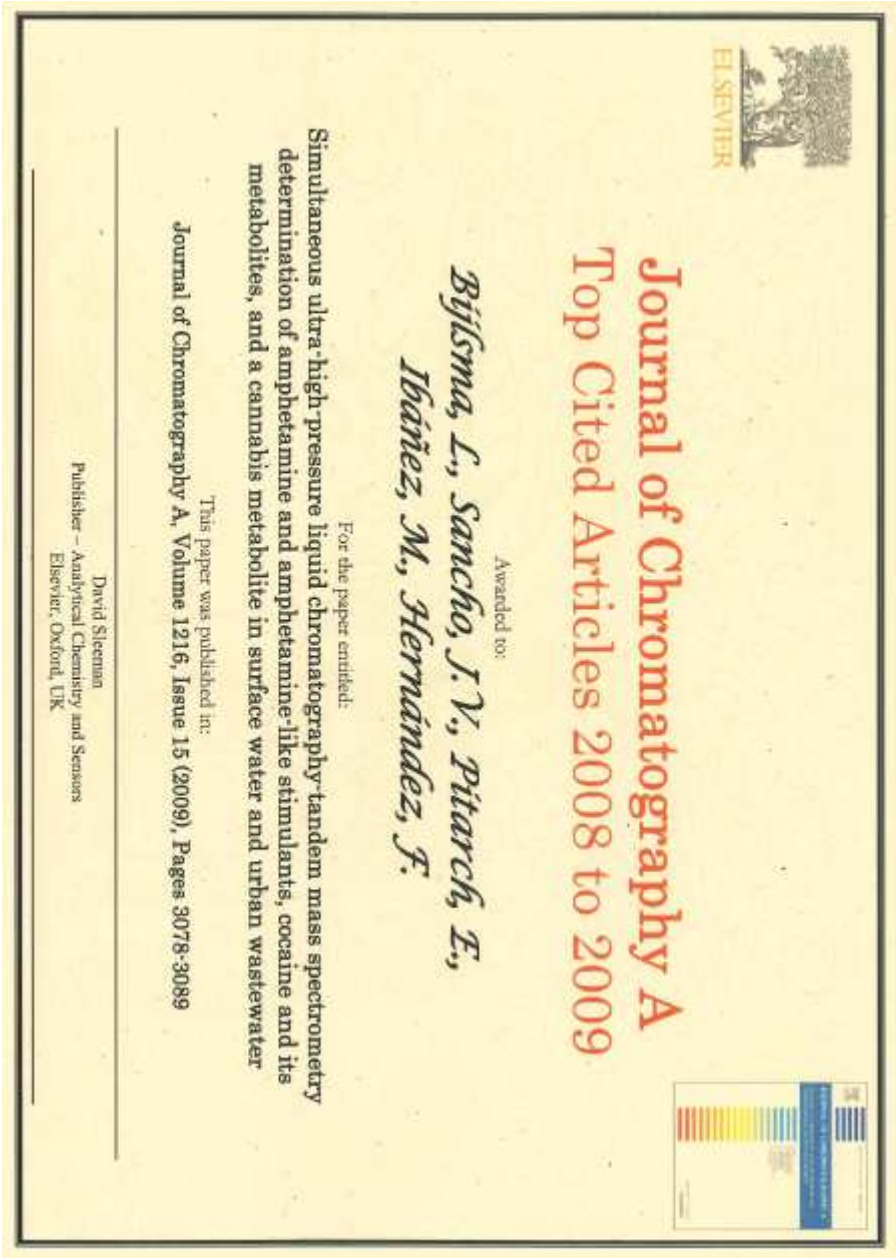
[26] D.T. Stafford, Editorial(2nd ed.)AACC Press, Washington, DC (2003) p. 89

[27] SANCO/2007/3131, Method validation and quality control procedures for pesticide residues analysis in food and feed.

(http://ec.europa.eu/food/plant/protection/resources/qualcontrol_en.pdf).

[28] Guidance Document on Residue Analytical Methods, SANCO/825/00 rev. 7 (http://ec.europa.eu/food/plant/protection/resources/guide_doc_825-00_rev7_en.pdf).

The Elsevier 2010 award as being one of the top 50 articles with more citations in the period 2008-2009



Chapter 2.2.2, scientific article 2

Improvements in analytical methodology for the determination of frequently consumed illicit drugs in urban wastewater

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RESEARCH PAPER

Improvements in analytical methodology for the determination of frequently consumed illicit drugs in urban wastewater

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Abstract

Rapid and sensitive analytical methodology based on ultra high-performance liquid chromatography-tandem mass spectrometry has been developed for the determination of widely consumed drugs of abuse (amphetamines, MDMA, cocaine, opioids, cannabis and ketamine) and their major metabolites in urban wastewaters. Sample clean-up and pre-concentration was performed by a generic off-line SPE procedure using Oasis HLB. Special effort was made to incorporate amphetamine, which was found highly problematic in the wastewater samples tested, including an additional clean-up with Oasis MCX SPE and dispersive primary secondary amine. Correction for possible SPE losses or degradation during storage was made by the use of isotope-labelled internal standards (ILIS), available for all compounds, which were added to the samples as surrogates. Although ILIS were also efficient for matrix effects correction, the strong ionization suppression observed was not eliminated; therefore, a four-fold dilution prior to SPE was applied to influent wastewaters and a low injection volume was selected (3 μL), in order to reach a compromise between matrix effects, chromatographic performance and sensitivity. The method was validated at 25 and 200 ng L^{-1} (effluent), and 100 and 800 ng L^{-1} (influent), obtaining limits of quantification (i.e. the lowest level that the compound can be quantified and also confirmed with at least two MS/MS transitions) between 0.4–25 ng L^{-1} (effluent) and 2–100 ng L^{-1} (influent). The applicability of the method was demonstrated by analysis of 14 influent and 14 effluent wastewater samples collected over 2 weeks in Castellón (Spain) within a European collaborative study.

Keywords

Drugs of abuse - Triple quadrupole - Ultra high-performance liquid chromatography - Sample dilution - Matrix effects - Urban wastewater

1. Introduction

Investigation of drugs of abuse (DOA) in the aqueous environment has become a prominent topic over the recent years. Data obtained from quantitative analysis of urban wastewater have been used to estimate drug consumption at local, national and international level. This is considered a promising approach in the field of epidemiological research on illicit drugs use and drug addiction [*1–3*], which offers complementary evidence-based, real-time information to existing survey-based studies. However, uncertainties of the different steps associated to this approach (e.g. sampling of wastewater, selection and stability of biomarkers, analytical measurements, back-calculation methods and estimation of population size) need to be tested and evaluated [*4*]. This is important in order to obtain realistic data and propose a uniform protocol which allows comparison of data between different locations and laboratories. Moreover, the results from both influent and effluent wastewater analysis have allowed the evaluation of removal efficiencies at wastewater treatment plants (WWTPs) and the estimation of discharges to receiving surface waters [*5–7*]. Detailed ecotoxicological studies should be performed to assess the potential negative effects associated to the presence of DOAs in the aquatic ecosystem [*8*].

Reliable concentration data obtained through extensive monitoring studies are required in all these studies. To this aim, sensitive and accurate analytical methodologies are necessary to determine DOA in water at sub-ppb levels (ng L^{-1}). High resolution mass spectrometry (HRMS) is a powerful technique for identification purposes and has been recently used for quantification purposes [*9*, *10*]. However, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is normally applied in this field due to its excellent sensitivity, selectivity and robustness [*11–17*].

In most LC-MS/MS methods reported, two transitions in selected reaction monitoring (SRM) mode are acquired, the most sensitive commonly used for quantification and the other for confirmation. However, a critical confirmation parameter, the ion ratio between both recorded transitions, is often unreported or not discussed despite the reliable identification of the compounds detected is of outstanding importance to avoid false positives or false negatives [*18*, *19*].

It is well known that one of the main limitations of LC-MS/MS is the existence of matrix effects, which make quantification and confirmation problematic especially at very low analyte concentrations. There are different approaches to compensate for matrix effects in multi-residue analysis each having advantages and drawbacks [*20*, *21*]. In the determination of DOA in wastewaters, the use of analyte isotopically labelled internal standards (ILIS) is the preferred approach to compensate for matrix effect. In addition, if ILIS are used as surrogates, i.e. added to the water samples just after sample collection, they can also compensate for errors related to sample pre-treatment (e.g. solid-phase extraction (SPE) step) and even to degradation during storage [*4*, *12*]. But even when analyte ILIS are available, a reduction of matrix effects may be necessary to improve precision and robustness in these complex-matrix samples. A simple approach is the dilution of the sample as demonstrated in other applied fields [22]. Although complete elimination of matrix effects would be desirable, the required dilution might shift the LOQs to unacceptable levels. Nevertheless, moderated sample dilution can be beneficial in two ways (1) improvement of the chromatographic performance of the analysis; (2) less amount of sample load may even lead to an increased signal, resulting in better sensitivity. Despite its simplicity, sample dilution to reduce matrix effects has very little been applied in the analysis of DOA in wastewater [*23*, *24*], surely because it is necessary that the LC-MS/MS instrument used needs to have excellent performance in terms of sensitivity.

In this work, improved LC-MS/MS methodology is developed for the simultaneous and sensitive determination of illicit drugs of abuse most frequently found in urban wastewaters, including parent compounds and major metabolites. The selected compounds were amphetamine, methamphetamine, MDMA (ecstasy), cocaine, heroin, 6-MAM (the minor but exclusive metabolite of heroin) morphine, codeine, ketamine, and the main metabolites of cocaine and cannabis, benzoylecgonine and THC-COOH, respectively. The present study aimed at a confident quantification and identification at low analyte concentrations, with special emphasis for amphetamine and THC-COOH. Sample pre-concentration was made by SPE using Oasis HLB, which was able to efficiently retain all the analytes. With the use of this generic/universal sorbent, we pursued to improve the scope of the method, and to reduce analysis time

and costs, as the same SPE sample extracts could be used for screening by LC-HR MS and/or investigation of other emerging contaminants such as pharmaceuticals. Sample extracts were injected in a state-of-the-art ultra high-performance liquid chromatography (UHPLC)-MS/MS with triple quadrupole (QqQ) analyser for rapid (chromatographic run <6 min) and more sensitive determination. A detailed study on the amount of sample loaded, by injecting less volume into the LC-MS/MS system, and/or by diluting the wastewater influents, with the objective of minimizing matrix effects and improving chromatographic performance, has been made. Furthermore, a critical discussion on the problems observed for amphetamine analysis has been also made. After validation, the developed method has been applied to the analysis of influent and effluent wastewater samples collected along 2 weeks from the WWTP of Castellón (Eastern Spain) within a wide European collaborative study.

2. Experimental

2.1. Chemicals and materials

The selection of the DOA was agreed upon for a collaborative comparison study project between 16 research institutes with the involvement of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). In total, 11 drugs and/or main metabolites were selected: amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy), cocaine, benzoylecgonine (BE), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), heroin, morphine, 6-monoacetylmorphine (6-MAM), codeine and ketamine. Reference standards of these compounds were purchased from Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol, acetonitrile or as salt. Isotopically labelled analogues used for quantification were: amphetamine-d_6, methamphetamine-d_5 MDMA-d_5, cocaine-d_3, benzoylecgonine-d_3 (BE-d_3), THC-COOH-d_3, heroin-d_9, morphine-d_3, 6-MAM-d_6, codeine-d_6 and ketamine-d_4. All ILIS were purchased from Cerilliant as solutions in methanol or acetonitrile. HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN), ammonium acetate, formic acid (>98 %) and primary secondary amine (PSA, 40–60 µm) sorbent were acquired from Scharlau

(Barcelona, Spain). HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA).

Standard stock solutions of each compound were prepared at 100 mg L^{-1} in MeOH or ACN. Intermediate solutions (10 mg L^{-1}) were prepared by diluting the stock solution ten times with MeOH. Infusion solutions of individual standards were prepared at a concentration of 1.5 mg L^{-1} in MeOH:water (50:50, v/v). Mixed working solutions containing all analytes were prepared from intermediate solutions by appropriate dilution with Milli-Q water, and were used for preparation of the calibration standards, internal quality controls and also for spiking samples in the validation study.

Individual stock solutions of isotope-labelled standards were prepared in MeOH or ACN at a concentration of 10 mg L^{-1}. A mixed standard working solution at 100 µg L^{-1} was prepared in water and was used as surrogate internal standard.

All standard solutions were stored in amber glass bottles at −20 °C.

SPE cartridges used were Oasis HLB 3 cm^3 (60 mg) and Oasis MCX 6 cm^3 (150 mg) from Waters (Milford, MA, USA).

2.2. Instrumentation

A Waters Acquity UHPLC system (Milford, MA, USA) was interfaced to a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK) equipped with T-Wave devices and an electrospray ionization interface (ESI) operated in positive-ion mode. Chromatographic separation was carried out using an Acquity UPLC BEH C_{18} column, 1.7 µm, 50 mm × 2.1 mm (i.d.) (Waters) at a flow rate of 0.3 mL min^{-1}. The column was kept at 40 °C and the sample manager was maintained at 5 °C. Mobile phase consisted of water with 5 mM ammonium acetate and 0.01 % formic acid (solvent A) and MeOH (solvent B). The percentage of MeOH changed linearly as follows: 0 min, 10 %; 3 min, 90 %; 3.5 min, 90 %; 3.6 min, 10 %; 6 min 10 %, equilibration of the column. Cone gas as well as desolvation gas was dry nitrogen. The cone gas and the desolvation gas flows were set to 250 and 1200 L h^{-1}, respectively. For operation in the MS/MS mode, collision gas was argon 99.995 % (Praxair, Madrid, Spain)

with a pressure of 4×10^{-3} mbar in the collision cell (0.15 mL min^{-1}). Other parameters optimized were: capillary voltage, 3.0 kV; source temperature, 150 °C and desolvation temperature, 650 °C. Dwell times of 0.01 s/transition were selected.

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

2.3. Matrix effect evaluation

Seven mixed working solutions containing all analytes were prepared from intermediate solutions at different concentrations ranging from 0 to 500 µg L^{-1} in MeOH:water (10:90, v/v). Exactly 25 µL of each mixture and 25 µL of a mixed ILIS working solution at 40 µg L^{-1} were added to a separate volume of 450 µL of a "blank" influent wastewater extract in order to evaluate matrix effects. The resulting concentrations of matrix-matched standards were 0, 0.5, 1, 2.5, 5, 10 and 25 µg L^{-1} (concentration ILIS: 2 µg L^{-1}). This procedure was repeated with the same extract diluted with Milli-Q water (1:2, 1:4, 1:8, 1:20 and 1:80) before fortification and with MeOH:water (10:90, v/v) to obtain calibration curve in solvent as reference. In this way, the effect of diluting the influent sample over matrix effects was tested.

2.4. Analytical procedure

Prior to solid-phase extraction, 100 mL effluent wastewater (EWW) and 100 mL four-fold diluted influent wastewater (IWW) samples were spiked with a mixed surrogate ILIS and subsequently vacuum filtered through 0.45 µm mixed cellulose ester membrane filters (Whatman, Dassel, Germany). The final concentration in sample for each ILIS was 20 ng L^{-1}. SPE was performed using Oasis HLB cartridges that were conditioned by washing and rinsing with 6 mL of MeOH and 6 mL of Milli-Q water. Samples were percolated through the cartridges by gravity (flow rate around 3 mL min^{-1}), and vacuum dried for approximately 15 min. Analytes were eluted using 5 mL of MeOH. The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen and reconstructed in 1 mL MeOH:water (10:90, v/v). Analyses were performed by injecting 3 µL of the final extract in the UHPLC-MS/MS system.

In the specific case of amphetamine, an additional clean-up using Oasis MCX cartridges was necessary. The Oasis MCX cartridges were conditioned with 6 mL of MeOH, 3 mL of Milli-Q water and 3 mL of acidified water (pH 2). An aliquot of 0.5 mL of the HLB extract (in MeOH:water 10:90) was 10 times diluted with water, acidified (pH 2) and percolated through the cartridges by gravity (flow rate around 3 mL min^{-1}). Then, cartridges were washed with 5 mL of acidified MeOH (pH 2) and vacuum dried for approximately 15 min. Analytes were eluted using 8 mL of a 2 % ammonia solution in MeOH. The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen and reconstructed in 0.5 mL MeOH:water (10:90, v/v). After reconstruction around 10 mg of PSA (a spatula tip) was added to the extract, centrifuged and 3 µL of the supernatant was injected in the UHPLC-MS/MS system for determination of amphetamine. A graphical workflow of the analytical procedure is presented in **Fig. 1**.

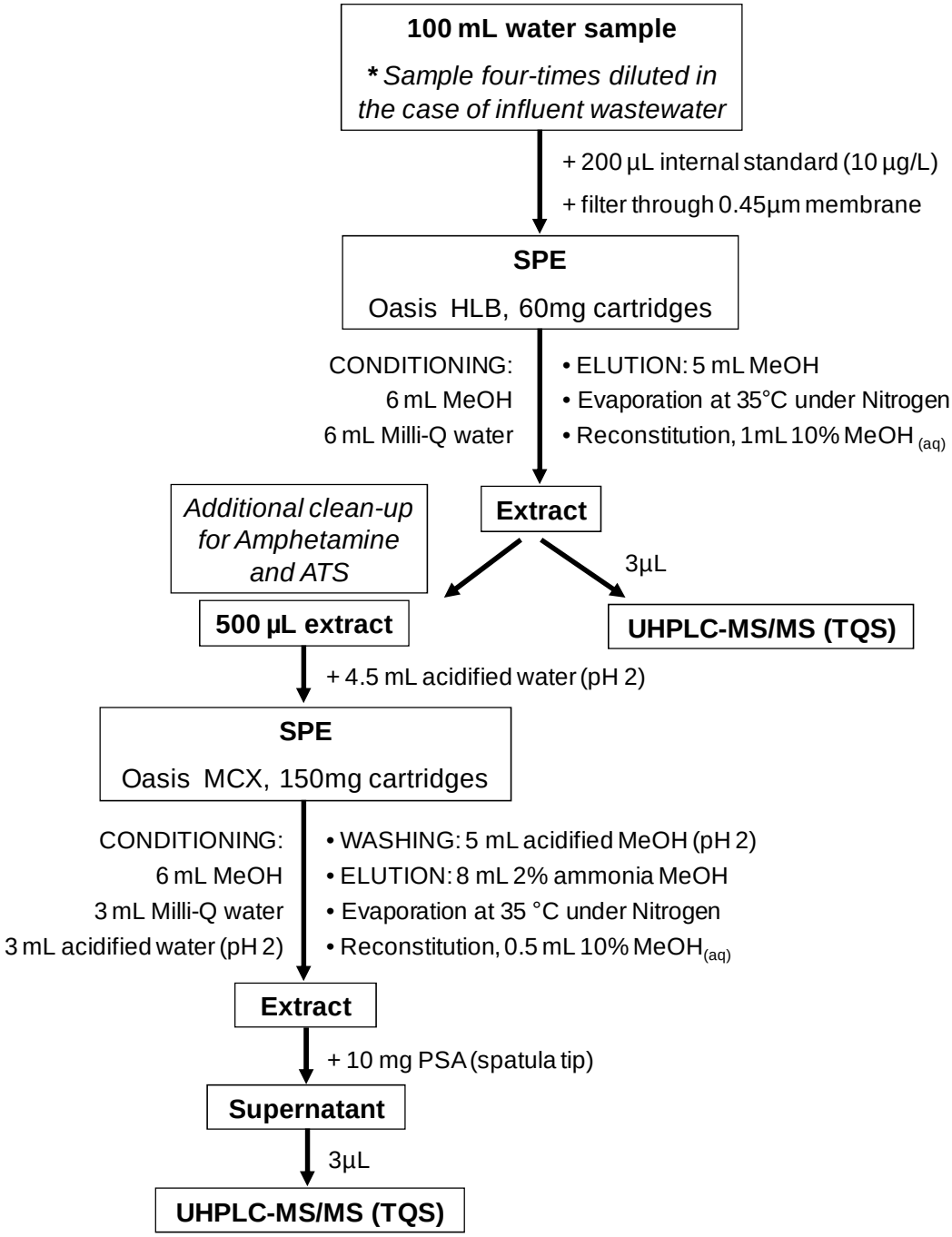

Figure 1. Graphical workflow of the analytical procedure.

2.5. Method validation

The performance of the method was evaluated in terms of linearity, limits of quantification, accuracy and precision.

Linearity was studied by analysing standard solutions in solvent in triplicate at six concentration levels ranging from 0.5 to 25 μg L^{-1}. Satisfactory linearity using weighted (1/X) least squares regression was assumed when the correlation coefficient (r) was >0.99, based on analyte/internal standard peak areas.

Limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio of 10 using the quantification transition, from SRM chromatograms of samples spiked at the lowest validation level tested. In addition, at least one of the confirmation transitions needed to show an S/N ratio of 3.

Instrumental limit of detection (LOD) was estimated for S/N = 3 from the chromatograms of the standards in solvent at the lowest concentration tested in the calibration curve.

Relative recoveries (%) of the whole procedure were evaluated by analysing influent and effluent wastewater samples spiked at different concentrations (25 and 200 ng L^{-1} in EWW; 100 and 800 ng L^{-1} in IWW). A mixed surrogate ILIS (20 ng L^{-1}) was added to compensate for matrix effects and possible losses during sample preparation. All recovery experiments were performed in quintuplicate (n = 5) for each type of water sample tested and for each spiking level. Accuracy (estimated by means of recovery experiments) between 70 and 120 % and precision (expressed as repeatability in terms of relative standard deviation (RSD)) < 20 % were considered as satisfactory.

2.6. Samples

In total, 14 influent and 14 effluent urban wastewater samples were taken from a WWTP of the province of Castellón (Eastern Spain). The WWTP investigated is equipped with conventional activated sludge secondary treatment and tertiary nitrogen and phosphate removal. The estimated residence time of the water in the WWTP is 24 h.

The 24 h composite samples (flow dependent, starting and finishing time (9:00 am to 9:00 am)) were collected over 14 consecutive days in April/May 2012, for influents starting on Wednesday April 18th and ending on Wednesday May 2nd and for corresponding effluents starting on Thursday April 19th and ending on Thursday May 3rd. All 28 samples were collected in polyethylene high density bottles and directly transported to the laboratory. Upon reception in the laboratory, samples were fortified with a mixed surrogate ILIS, filtered and immediately stored in the dark at −20 °C until analysis.

3. Results and discussion

3.1. UHPLC and MS/MS optimization

Infusion solutions of individual standards were used to optimize MS conditions and to select MS/MS transitions for each target analyte and ILIS. The best results in terms of sensitivity were obtained with ESI operating in positive ionization mode, using the protonated molecule $[M+H]^+$ as precursor ion. Working under SRM mode, the most sensitive transition (in terms of signal-to-noise ratio) was selected for quantification whereas the two other transitions were acquired for confirmation. MS/MS parameters established for the SRM acquisition mode, as well as the transitions selected, instrumental LODs and retention times are listed in **Table 1**. This table also shows the average Q/q values obtained from the calibration standards. The RSD's for Q/q ratios illustrate whether these ratios are considered to be concentration dependent or not (i.e. an RSD < 10 % would indicate little variation of the Q/q values over the concentration range 0.5 to 25 µg L^{-1}).

In total, 44 SRM transitions, three SRM transitions for each compound and the quantification transition for each individual ILIS were acquired. In order to acquire the maximum number of data points across each chromatographic peak (at least ten points-per-peak) without evident resolution and/or sensitivity losses, short dwell times were selected. Using our fast-acquisition triple-quadrupole mass analyser, dwell times as low as 0.003 s per transition could be automatically setup allowing satisfactory peak shape and sensitivity.

Table 1: UHPLC-MS/MS parameters established for the SRM acquisition mode (quantification (Q) and confirmation (q_1 and q_2) transitions).

Compound	R_t (min)	LOD (fg)	Q transition	CV (V)	CE (eV)	q_1 transition	CE (eV)	q_2 transition	CE (eV)	Q/q_1 (RSD %)	Q/q_2 (RSD %)
Amphetamine	1.87	950	136 > 119	20	10	136 > 91	20	119 > 91	10	1.2 (10)	1.6 (8)
Amphetamine-d_6	1.86		142 > 125	20	10						
Methamphetamine	1.87	200	150 > 119	35	10	150 > 91	20	150 > 65	35	0.5 (5)	28.8 (16)
Methamphetamine-d_5	1.86		155 > 121	35	10						
MDMA	1.86	130	194 > 163	30	15	194 > 105	25	194 > 77	40	2.0 (3)	7.9 (7)
MDMA-d_5	1.86		199 > 165	30	15						
Cocaine	2.28	130	304 > 182	30	20	304 > 82	30	304 > 77	50	2.9 (8)	10.0 (9)
Cocaine-d_3	2.27		307 > 185	30	20						
BE	2.05	110	290 > 168	40	20	290 > 105	30	290 > 82	30	2.8 (10)	5.1 (8)
BE-d_3	2.05		293 > 171	40	20						
THC-COOH	4.03	600	345 > 193	40	25	345 > 299	20	345 > 327	15	0.5 (6)	0.3 (8)
THC-COOH-d_3	4.02		348 > 196	40	25						
Heroin	2.19	110	370 > 268	35	25	370 > 165	30	370 > 211	50	0.8 (7)	1.3 (7)
Heroin-d_9	2.17		379 > 272	35	25						
Morphine	1.12	230	286 > 152	40	40	286 > 201	50	286 > 128	25	0.5 (3)	1.3 (9)
Morphine-d_3	1.12		289 > 152	40	40						
6-MAM	1.69	50	328 > 165	40	40	328 > 211	40	328 > 181	25	1.4 (7)	3.2 (4)
6-MAM-d_6	1.69		334 > 165	40	40						
Codeine	1.53	30	300 > 215	20	25	300 > 199	30	300 > 162	45	1.6 (6)	145.6 (17)
Codeine-d_6	1.51		306 > 218	20	25						
Ketamine	2.18	40	238 > 125	20	25	238 > 179	15	238 > 207	15	3.4 (11)	5.0 (5)
Ketamine-d_4	2.17		242 > 129	20	25						

R_t retention time, LOD instrumental limit of detection, Q quantification, q confirmation, CV cone voltage, CE collision energy

Yet, for the most polar and non-polar compounds, morphine (t_R, 1.12 min) and THC-COOH (t_R, 4.03 min), respectively, the number of data points-per-peak could be easily increased by splitting their SRM transitions into two separate elution-time windows. In this way, for example, a better calibration fit was achieved for morphine.

Different mobile phase compositions were tested in order to get good chromatographic separation with satisfactory chromatographic peaks for all selected compounds. Formic acid (0.01 %) was added to both water and MeOH mobile phase solvents, favouring the formation of the protonated molecule. Sensitivity and chromatographic performance improved for all compounds, except for THC-COOH. Therefore, formic acid was not added to MeOH so that its effect on this late-eluting compound was diminished. Furthermore, the addition of ammonium acetate to the water phase allowed improving the peak shapes for several target compounds especially when analysing the wastewater samples. A gradient consisting of water 5 mM ammonium acetate and 0.01 % formic acid (solvent A) and MeOH (solvent B) was finally chosen as mobile phase.

3.2. Sample handling and preparation

Two types of cartridges were considered: Oasis MCX and Oasis HLB. After preliminary experiments with Oasis HLB using both effluent and influent wastewater, we optimized the procedure as described in "Analytical procedure", obtaining satisfactory absolute recoveries (70–120 %) for all target analytes. Oasis HLB was selected for subsequent validation owing to the efficiency of this polymeric sorbent for extraction of a wide range of compounds, with rather different physico-chemical characteristics, using a simple and generic protocol [25]. The development of a generic sample preparation procedure was of interest for us as the same sample extracts might also be used for wide-scope screening of DOA, pharmaceuticals/antibiotics, personal-care-products and other emerging contaminants based on UHPLC-QTOF MS analysis [26]. This is highly beneficial for the laboratory in terms of efficiency and time and cost reduction.

However, for amphetamine, we observed some difficulties related to sensitivity and identification capability. The reliable determination of this analyte was problematic at

lower concentrations even using ILIS (a troubleshoot discussion on the problems observed for amphetamine analysis is described in the next section). Special effort, i.e. additional clean-up by Oasis MCX and PSA, was done to incorporate amphetamine in our method as data on the samples analysed were expected to be used in a wide collaborative research on DOA consumption in Europe.

3.3. The particular case of amphetamine

Strong matrix interferences, especially in influent wastewater, made the determination of amphetamine highly problematic. Difficulties related to sensitivity and identification capabilities were found, a fact that was not observed before [24]. A possible explanation of the lower sensitivity for amphetamine and/or increased effect for certain matrix interferences might come from the newly designed "Step-wave" ion guide. This Step-wave device, in theory, maximizes ion transmission from the source to the mass analyser as well as in the collision cell, providing an enhancement of the overall signal-to-noise ratio. This was especially true for THC-COOH, which showed improved sensitivity for all transitions compared to the results obtained using an older triple quadrupole analyser (TQD, Waters Micromass, Manchester, UK), without Step-wave device [24]. On the contrary, amphetamine showed a significant decrease in sensitivity for all transitions, something which was not observed for other amphetamine-like stimulants such as methamphetamine and MDMA. Furthermore, lower sensitivity for some small molecular-size compounds (e.g. aldicarb and propham) was also observed in our laboratory when developing a multi-residue pesticide method (data not shown), an indication that the Step-wave technology might have negative effects on sensitivity for small molecules. In order to improve sensitivity, some adjustments to the step-wave of the TQS system were made, but without the desired outcome.

In addition to the above-mentioned negative effect of the Step-wave device, one must take into account that using the "universal" HLB sorbent, although beneficial for the scope of the method might however lead to an increase of matrix components that remained retained in the cartridge. This became more relevant considering the lower sensitivity obtained for amphetamine. Therefore, an additional clean-up was tested. After several experiments, Oasis MCX SPE cartridges and PSA were selected to

remove matrix interferences trying to reach the desired low LOQ values. **Figure 2** shows four SRM transitions selected for amphetamine. A standard solution and spiked influent wastewater extracted by solely Oasis HLB and by Oasis HLB followed by Oasis MCX and PSA were compared. As it can be seen from this figure, matrix interferences were considerably removed when performing an additional clean-up. Furthermore, one of the SRM transitions (136 > 65) showed poor sensitivity; therefore another transition (119 > 91) promoted by in-source fragmentation was selected to have additional confirmation.

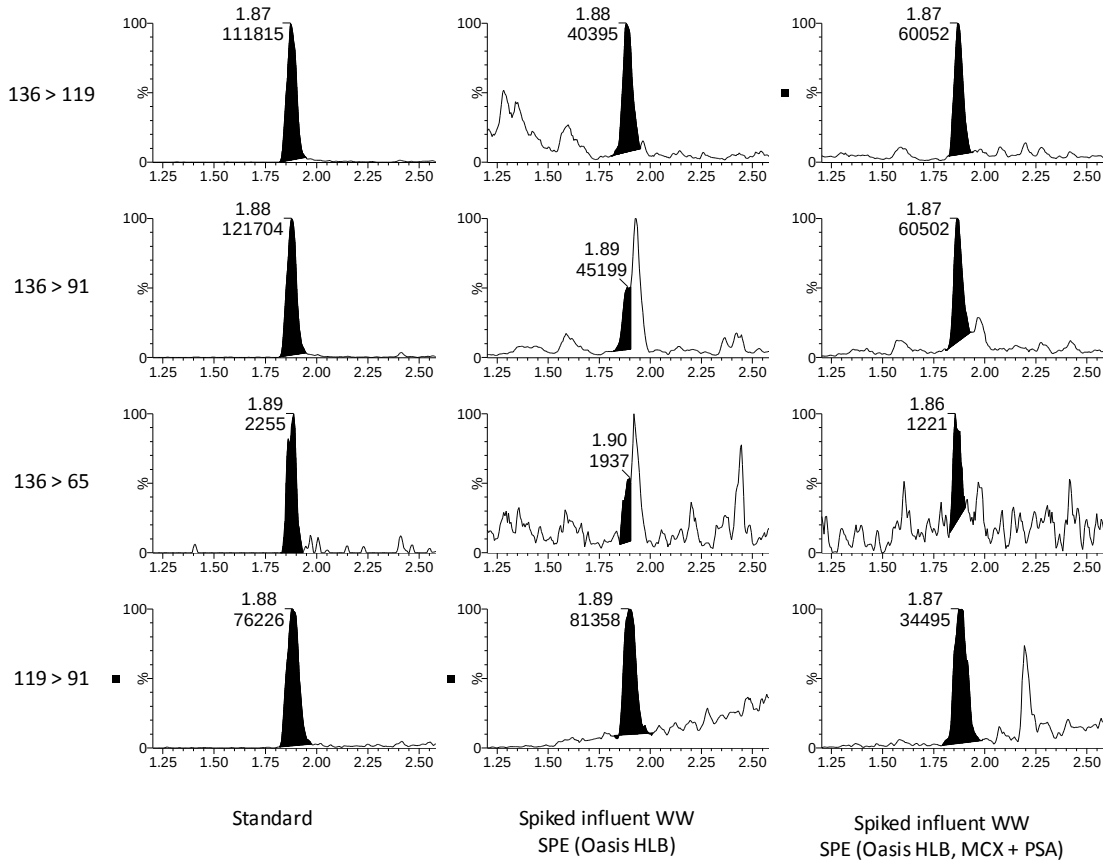

Figure 2. UHPLC-MS/MS chromatograms (four SRM transitions of amphetamine) for a standard solution and spiked influent wastewater extracted by solely Oasis HLB and by Oasis HLB followed by Oasis MCX and PSA (concentration 2.5 μg L^{-1}).

Occasionally, in some of the samples analysed, this clean-up procedure slightly improved the chromatographic performance of methamphetamine and MDMA too, and was thus selected as general procedure for these compounds.

3.4. Reduction of matrix effects

Compensation for matrix effects was achieved using analyte ILIS, available for each compound. However, when matrix components overload the LC-MS/MS system, appropriate sample dilution may improve the detectability of the analytes as well as the overall performance of the method.

A preliminary study demonstrated that dilution of effluent wastewater would not be advantageous (data not shown). Therefore, the effect of dilution was tested only for influent wastewater, as the high complexity and elevated organic matter content of this type of samples, allow expecting notable improvements if matrix effects were minimized. Standards in influent wastewater applying different dilution factors and standards in solvent were prepared in order to evaluate the dilution needed for satisfactory chromatographic performance and LOQ of each analyte. Instead of diluting the samples prior to the SPE step, dilution was carried out using a pooled homogenized SPE extract of the same influent wastewater. This reduced the number of experiments, but also placed us in the worst-case scenario, as the raw sample was loaded instead of the diluted samples, which would have alleviated the potential negative effect of the matrix components on cartridge retention (e.g. overloading).

The influence of matrix concentration on calibration curves was similar for all DOA studied. As an example, the calibration curves of the 6-MAM obtained from the standards in solvent (no matrix effect) and from standards in influent wastewater at different dilutions showed notable differences in slopes indicating a significant signal suppression (**Fig. 3**). In the worst-case scenario (i.e. non diluted sample), the matrix effect calculated from the slope (IWW/solvent × 100 %) was 7 %, which illustrate the strong ion suppression. Similar behaviour was observed for the other selected compounds.

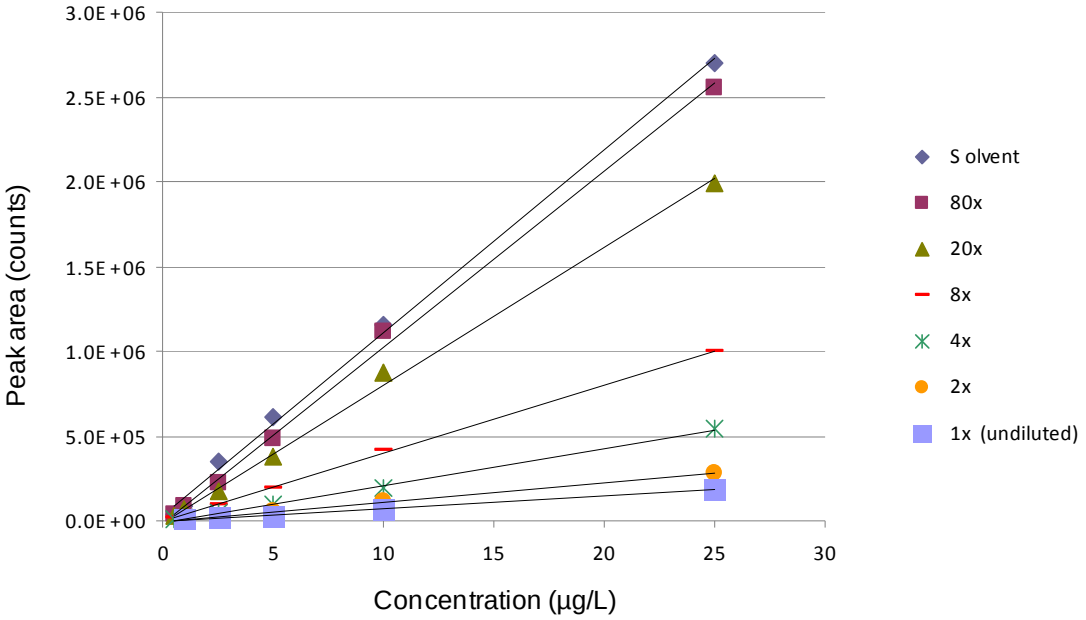

Figure 3. Calibration curves of 6-MAM in solvent and in influent wastewater in the concentration range 0.5–25 µg L^{-1}. The influent wastewater extract was diluted in the range 1 (undiluted) to 80 times.

Figure 4 shows peak areas of each analyte divided by the dilution factor (DF) plotted against the DF. As can be observed, relative responses of opioids (i.e. heroin, morphine 6-MAM and codeine) decreased when increasing the DF, suggesting low matrix effects. In this case, dilution would result in undesirable higher LOQs. However, for amphetamines and ketamine the opposite seemed to occur: relative responses initially increased when samples were diluted, showing a beneficial effect of dilution that led to the minimization of the strong matrix effects observed for these compounds. A four-time dilution factor appeared as a good compromise for the compounds selected as higher dilutions showed no further benefit.

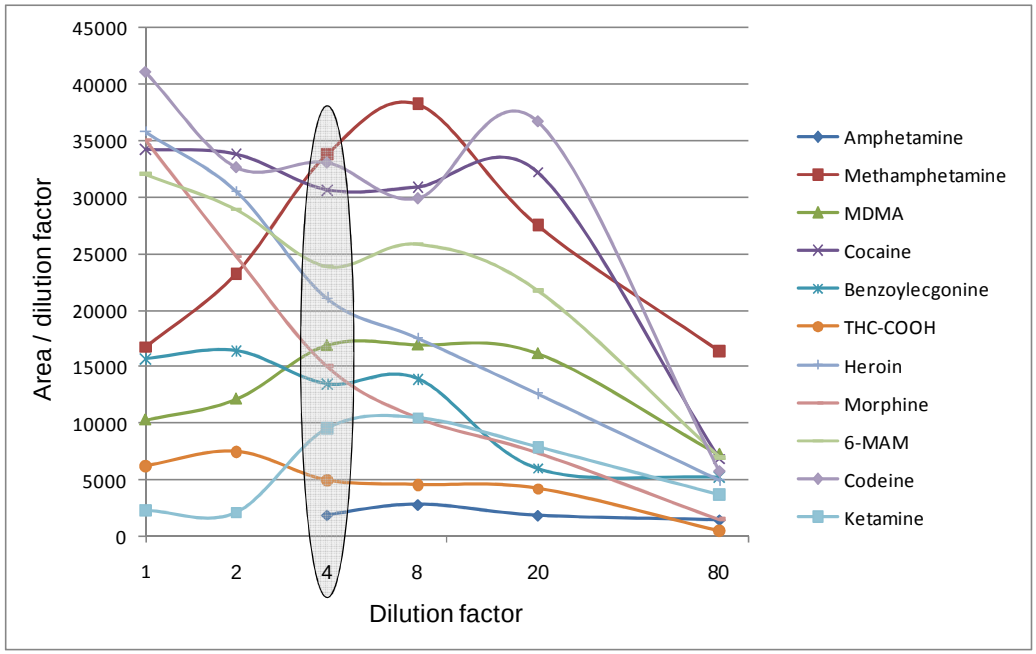

Figure 4. Responses of illicit drugs and metabolites of matrix-matched standards (5 µg L^{-1}) applying different dilution factors (DF) in the range from 1 (undiluted) to 80 times dilution. Note: responses of morphine and 6-MAM were divided by 2 and responses of cocaine and BE were divided by 10 in order to present a clarifying graphic.

The chromatographic performances were also evaluated. **Figure 5** shows UHPLC-MS/MS chromatograms (quantification transition) of MDMA, cocaine, THC-COOH, 6-MAM and ketamine of an undiluted and a four-fold diluted influent wastewater SPE extract spiked at 10 and 2.5 µg L^{-1}, respectively. The spiked concentrations in both SPE extracts would correspond to 100 ng L^{-1} of illicit drugs and metabolites in the sample. Chromatographic peaks shapes were similar for cocaine, THC-COOH and 6-MAM, but they considerably improved for MDMA and ketamine when diluting the sample four times, due to the less matrix injected in the chromatographic system. In this sense, it is worth mentioning that not only poorer peak shapes were observed, but also retention times of some compounds slightly shift in undiluted samples compared with that of the standard. Although most compounds fit within its tolerance (±2.5 %), retention times of cocaine (9.2 %) and heroin (8.2 %) were out of this criteria.

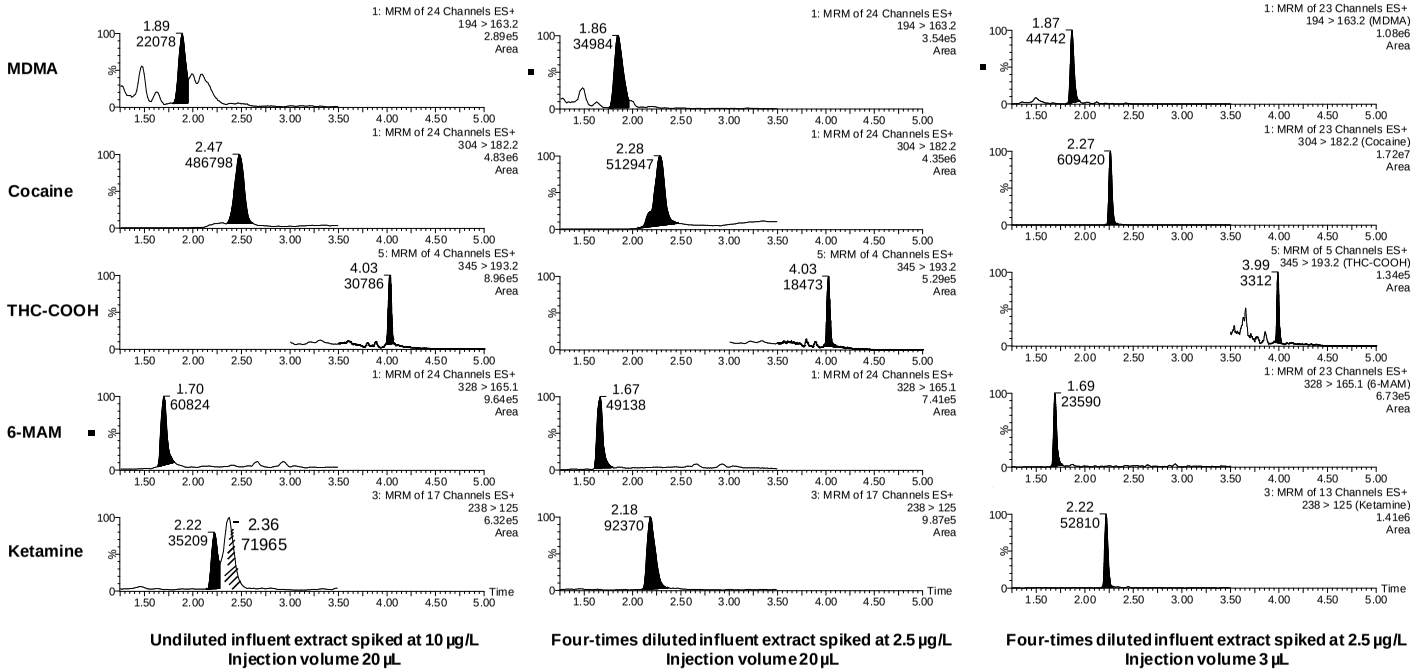

Figure 5. UHPLC-MS/MS chromatograms (quantification transition) for an undiluted and four-times diluted influent wastewater SPE extract spiked at 10 and 2.5 µg L^{-1}, respectively. The spiked concentrations would correspond to 100 ng L^{-1} of illicit drugs and metabolites in the wastewater sample

In order to further improve chromatographic performance of some analytes, injection of smaller sample volumes (20, 10, 5, 3 and 1 µL) was tested. **Figure 5** illustrates that reducing injection volume from 20 µL to 3 µL led to an improvement in chromatographic peak shape as well as peak area for MDMA and cocaine. Although the behaviour of THC-COOH would recommend injecting 20 µL extract of undiluted influent wastewater sample, an acceptable LOQ level could still be obtained.

Finally, a four-fold dilution of influent wastewater before SPE step and 3 µL of injection volume were considered as a good compromise to improve chromatographic peak shapes and detectability for the target compounds evaluated. These values were selected for further validation.

3.5. Method validation

The overall analytical procedure, as described in "Analytical procedure", was validated for the two types of urban wastewater studied in this work: effluent (**Table 2**) and influent (**Table 3**).

Good linearity was shown for six-point calibration curves in the range of 0.5 to 25 µg L^{-1} for all selected compounds, with correlation coefficients greater than 0.99.

The accuracy and precision of the method were estimated by means of recovery experiments, using "blank" wastewater samples spiked at two concentration levels (25 and 200 ng L^{-1} in EWW; 100 and 800 ng L^{-1} in IWW). True blank wastewater samples were not feasible to obtain; therefore EWW and IWW were previously analysed and those with lower DOA levels were selected as "blank" samples. Concentrations of target compounds found in these "blank" samples were subtracted from the spiked samples. All experiments were carried out in quintuplicate at each level.

In general, the results obtained for most compounds were satisfactory at both fortified levels, with recoveries between 70 and 120 % and precision (RSD) below 20 %. In influent wastewater (**Table 3**), cocaine and heroin showed relative high recoveries (124 and 128 %, respectively), but with good RSD (5 and 7 %, respectively) at the highest fortified level. Furthermore, accuracy and precision of BE, spiked at the lowest validated

concentration could not be calculated, due to high concentrations found in the "blank" sample (≈1,400 ng L^{-1}).

Table 2. Method validation in effluent wastewater (n = 5).

Compound	Recovery (RSD)		LOQ	Q/q_1 ratio dev [a]	Q/q_2 ratio dev [a]
	25 ng L^{-1}	200 ng L^{-1}	(ng L^{-1})	(%)	(%)
Amphetamine	97 (6)	94 (5)	25	4 (-12 to +3)	25 (-32 to -22)
Methamphetamine	97 (6)	94 (4)	20	6 (+3 to +12)	88 (-81 to -92)
MDMA	101 (4)	103 (4)	6	26 (+11 to +36)	14 (+5 to +20)
Cocaine	106 (4)	107 (2)	2	6 (0 to +13)	26 (-26 to -22)
BE	119 (8)	109 (7)	0.4	5 (-1 to +10)	38 (+33 to +43)
THC-COOH	109 (4)	117 (2)	7	2 (0 to +5)	7 (-1 to +14)
Heroin	105 (6)	101 (2)	6	8 (-14 to -3)	6 (-11 to -3)
Morphine	106 (8)	98 (3)	10	6 (-4 to +17)	7 (+5 to +10)
6-MAM	106 (5)	101 (2)	8	10 (+4 to +16)	10 (+6 to +16)
Codeine	108 (7)	100 (4)	2	8 (+4 to +14)	30 (-46 to -7)
Ketamine	108 (8)	112 (2)	3	9 (-16 to -2)	1 (-10 to +14)

[a]Average deviation and range (in %) of the experimental Q/q ratios obtained from the sample extracts spiked at 25 ng L^{-1} (n = 5) in relation to those calculated from standard solutions in solvent (see ***Table 1***)

Table 3. Method validation in influent wastewater (n = 5).

Compound	Recovery (RSD)		LOQ	Q/q_1 ratio dev [a]	Q/q_2 ratio dev [a]
	100 ng L^{-1}	800 ng L^{-1}	(ng L^{-1})	(%)	(%)
Amphetamine	77 (11)	98 (8)	100	0 (-11 to +9)	27 (10 to 37)
Methamphetamine	91 (4)	92 (6)	82	7 (-12 to +5)	74 (-77 to -72)
MDMA	102 (4)	102 (6)	30	7 (-1 to +14)	8 (-22 to +45)
Cocaine	115 (16)	124 (5)	5	8 (-12 to -3)	7 (-14 to -3)
BE	[b]	86 (12)	2	11 (-13 to -8)	4 (-7 to -2)
THC-COOH	105 (5)	115 (8)	60	10 (+5 to +16)	20 (+15 to +24)
Heroin	118 (4)	128 (7)	43	2 (-11 to +10)	0 (-7 to +7)
Morphine	93 (6)	92 (11)	21	8 (+6 to +10)	7 (+6 to +9)
6-MAM	86 (12)	83 (8)	37	11 (+8 to +16)	3 (-7 to +1)
Codeine	88 (19)	73 (8)	5	4 (-7 to 0)	56 (-84 to -3)
Ketamine	99 (2)	103 (8)	19	11 (-13 to -9)	2 (-6 to +9)

[a]Average deviation and range (in %) of the experimental Q/q ratios obtained from the sample extracts spiked at 100 ng L^{-1} (n = 5) in relation to those calculated from standard solutions in solvent (see ***Table 1***)
[b]Not calculated due to the high concentration found in the "blank" sample

The peak area ratios for the lowest validated concentration (worst case) were calculated between the quantification (Q) and confirmation (q_1 and q_2) transitions and compared with the average Q/q value obtained from the calibration standards in solvent. In some cases, the ion ratio fell outside the maximum deviation permitted (**Tables 2 and 3**). This might be due to the presence of matrix components sharing one of the two transitions, thus affecting the Q/q ratio. Therefore, the acquisition of more than two transitions is recommended to facilitate confirmation of the positives by testing the additional transitions acquired [*4*]. In this study, the identity of the compounds at the lowest validated concentration could be confirmed in all cases even with three transitions complying ion ratios and retention time within tolerances, except for amphetamine, methamphetamine and codeine which could be confirmed by two transitions, i.e. one ion ratio accomplishment.

The LOQs were estimated for a S/N ratio of 10 using the quantification transition, from chromatograms of "blank" samples spiked at the lowest validation level tested, and taking into account the analyte concentration found in the "blank". In addition, at least one of the confirmation transitions needed to show an S/N ratio of ≥3. Thus, a possible positive finding at that concentration level could also be confirmed according the European guidelines followed in this work [*18*]. LOQs ranged from 0.4 to 25 ng L^{-1} for EWW (**Table 2**) and from 1.6 to 100 ng L^{-1} for IWW (**Table 3**). Regarding BE in IWW, the LOQ was estimated from chromatograms of the quantified "blank" sample, without spiking the sample.

It is interesting to mention the difficulties around the determination of cannabis, i.e. its main metabolite THC-COOH, which surely is the most consumed illicit drug. The determination of this metabolite in sewage poses some analytical challenges. Its lower polarity compared with other illicit drugs makes it more difficult to include this compound in a multiclass LC-MS/MS method. Thus, the determination of DOA has been almost exclusively based on electrospray ionization, which usually provides better sensitivity, especially for the more polar compounds. As a result, poor LOQs are commonly reported for THC-COOH. In order to increase sensitivity, both positive [*7*, *9*, *15*, *24*] and negative [*6*, *10*, *16*] ionization modes have been tested, and the final

selection made mainly depending on the instrument used. The analytical limitations related to this compound have been widely recognized in the literature [9, 16, 24, 27]. Despite the difficulties, in the present paper the determination of THC-COOH was performed in ESI positive mode and the LOQs were rather satisfactory: 7 ng L^{-1} (effluent) and 60 ng L^{-1} (influent). At these low concentrations, not only quantification but also confirmation of its identity was feasible thanks to the acquisition of three MS/MS transitions and accomplishment of at least one ion ratio. As previously mentioned, this was facilitated by the use of a newly designed Step-wave ion guide incorporated in the LC-MS/MS instrument.

3.6. Application to real samples

The developed method was applied to the analysis of 24-h composite flow-dependent wastewater samples. Both influents and effluents were collected daily from the same WWTP over 14 consecutive days during April–May 2012. All 28 samples were analysed in triplicate.

In each sequence of analysis, the calibration curve was injected in duplicate, at the beginning and at the end. Internal quality controls (QCs), i.e. "blank" samples fortified at the two validated levels, were alternatively injected after every six injections. The sample sequences were considered as satisfactory as QCs relative recoveries ranged from 60 to 130 % for the illicit drugs detected.

Daily variance of mean concentrations (in ng L^{-1}) for illicit drugs detected in wastewater is illustrated in **Fig. 6**. In influents, mean concentrations ranged from 184 to 957 ng L^{-1} (cocaine), 945 to 2276 ng L^{-1} (BE), 501 to 916 ng L^{-1} (THC-COOH), 89 to 141 ng L^{-1} (morphine) and 348 to 560 ng L^{-1} (codeine). The highest mean concentration was observed for BE (2276 ng L^{-1}) which corresponded to Friday April 27th 2012 (in the case of BE, quantification of highly concentrated samples required an additional analysis after appropriate dilution of the SPE extract before injecting into the LC-MS/MS). An expected weekend increase of cocaine excretion, revealed by its main metabolite BE, was observed (**Fig. 6A**). The other drugs detected in influents (THC-COOH, morphine and codeine) did not show big variations suggesting that the

consumption of these drugs is quite constant over the week. In general, this is in agreement with the daily dynamics of illicit drugs found in other studies [*1*, *5*, *7*]. The absence of amphetamine, methamphetamine, MDMA, ketamine, heroin and its minor but exclusive metabolite 6-MAM might indicate that these drugs are not extensively consumed in Castellón.

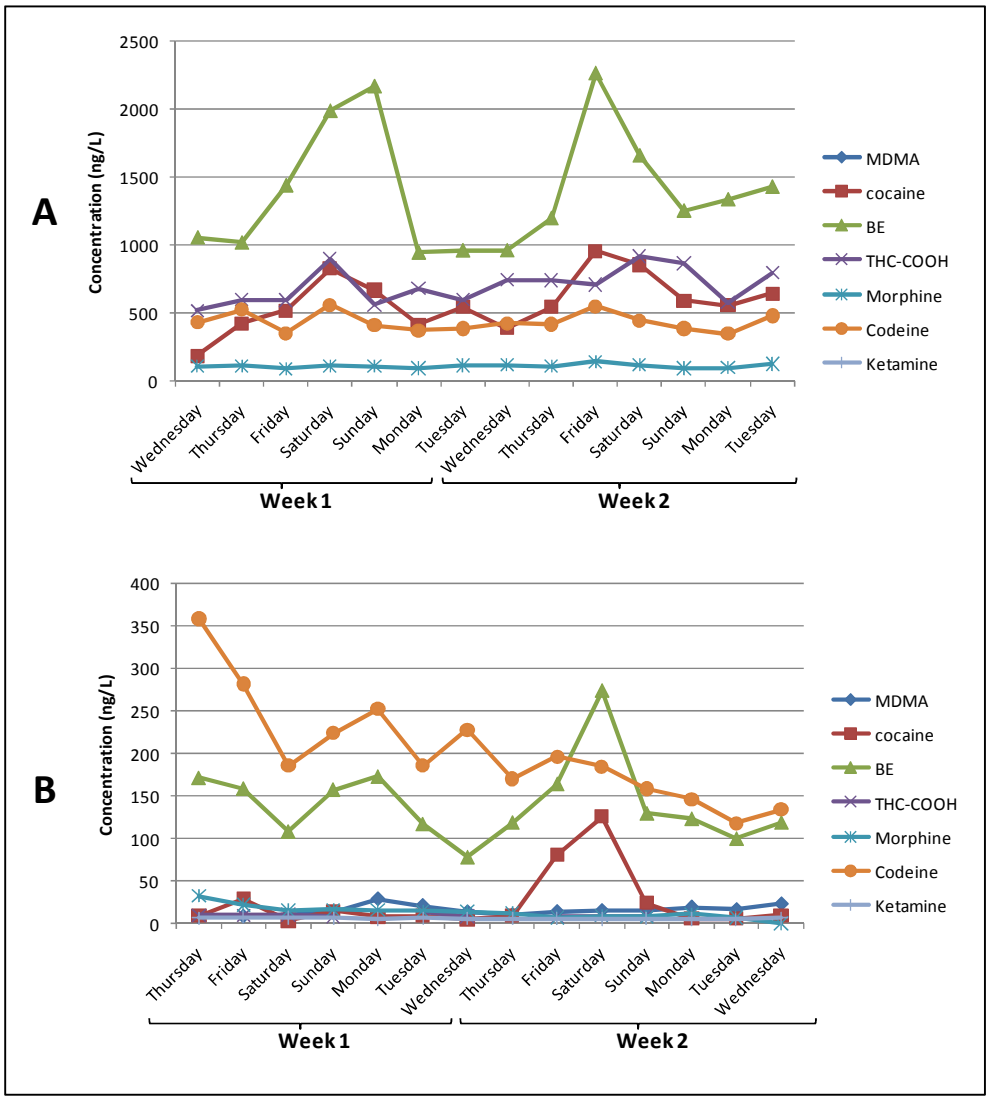

Figure 6. Daily variance of mean concentrations (n = 3) for illicit drugs detected in wastewater influents (A) and effluents (B) during 14 consecutive sampling days in Castellón de la Plana (Spain).

The five illicit drugs found in influents (cocaine, BE, THC-COOH, morphine and codeine) were also detected in their corresponding effluents (**Fig. 6B**), but at lower mean concentrations, suggesting removal by degradation or sorption of these substances in the WWTP. In addition to these five compounds, MDMA and ketamine were also detected in effluents, although at low concentrations (maximum concentrations of 28 and 7.0 ng L^{-1}, respectively). The detection of MDMA and ketamine in effluents and their absence in influents might be due to the difficulties of detection and quantification of low analyte levels in influents, despite the efforts made to decrease LOQs. Of interest is the pattern of codeine in effluent, which seems to slowly decline during the sampling period. However, no clear explanation can be given to this fact.

The detection, quantification at the low analyte concentrations, the satisfactory recoveries obtained for QC samples, and the safe identification by using up to three SRM transitions per compound, in both IWW and EWW samples, support the reliability of the analytical methodology developed in this article.

4. Conclusions

Modern analytical methodology based on the use of a state-of-the-art UHPLC-MS/MS with triple quadrupole mass analyser has been developed for the simultaneous quantification and confirmation of widely consumed and commonly detected drugs of abuse and major metabolites in urban wastewater. Sample pre-concentration was performed by a simple and generic off-line SPE procedure using Oasis HLB cartridges, which allows the use of the same sample extracts for wide-scope screening purposes (commonly based on LC-HR MS analysis). Special effort has been made to improve amphetamine analysis, including an additional clean-up using Oasis MCX SPE cartridges and PSA in this particular case. The cannabis metabolite THC-COOH was also of particular interest due to the analytical problems commonly observed in the determination of this compound in wastewaters. Thus, a notable improvement in sensitivity was reached in relation to the previous methodology applied at our laboratory. Matrix effects were reduced by injecting less sample volume and diluting the influent wastewater sample before SPE. This allowed to notably improve chromatographic peak shapes and detectability for several analytes. Analyte ILIS, available for all target compounds, were used for accurate quantification of the selected analytes in these complex matrices. The reliable identification of positive findings was assured by the acquisition of three SRM transitions per compound and the compliance of ion ratios. The latter was found in some cases problematic, mainly at low analyte levels. To this aim, the acquisition of more than two transitions is recommended to improve the reliability of identification particularly in those cases where one of the transitions seemed to be interfered. The developed method was applied to the analysis of drugs of abuse and their metabolites in influent and effluent wastewater collected over 14 consecutive days. The lower DOA concentrations commonly found in effluent samples suggested that a removal by degradation and/or sorption of these substances takes place in the WWTP.

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References

[1] Thomas KV, Bijlsma L, Castiglioni S, Covaci A, Emke E, Grabic R, Hernández F, Karolak S, Kasprzyk-Hordern B, Lindberg RH, Lopez de Alda M, Meierjohann A, Ort C, Pico Y, Quintana JB, Reid M, Rieckermann J, Terzic S, van Nuijs ALN, de Voogt P (2012) Sci Total Environ 432:432 - 439

[2] van Nuijs ALN, Mougel J-F, Tarcomnicu I, Bervoets L, Blust R, Jorens PG, Neels H, Covaci A (2011) Environ Int 37:612 - 621

[3] Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R (2008) Environ Health Perspect 116:1027 - 1032

[4] Castiglioni S, Bijlsma L, Covaci A, Emke E, Hernández F, Reid M, Ort C, Thomas KV, van Nuijs ALN, de Voogt P, Zuccato E (2013) Environ Sci Technol 47:1452 - 1460

[5] Bijlsma L, Emke E, Hernández F, de Voogt P (2012) Chemosphere 89:1399 - 1406

[6] Postigo C, López de Alda MJ, Barceló D (2010) Environ Int 36:75 - 84

[7] Terzic S, Senta I, Ahel M (2010) Environ Pollut 158:2686 - 2693

[8] van der Aa M, Bijlsma L, Emke E, Dijkman E, van Nuijs ALN, van de Ven B, Hernández F, Versteegh A, de Voogt P (2013) Water Res 47:1848 - 1857

[9] Bijlsma L, Emke E, Hernández F, de Voogt P (2013) Anal Chim Acta 768:102 - 110

[10] González-Mariño I, Quintana JB, Rodríguez I, González-Díez M, Cela R (2012) Anal Chem 84:1708 - 1717

[11] Baker DR, Kasprzyk-Hordern B (2011) J of Chromatogr A 1218:1620 - 1631

[12] Castiglioni S, Zuccato E, Chiabrando C, Fanelli R, Bagnati R (2008) Mass Spectrom Rev 27:378 - 394

[13] van Nuijs ALN, Castiglioni S, Tarcomnicu I, Postigo C, Lopez de Alda M, Neels H, Zuccato E, Barcelo D, Covaci A (2011) Sci Total Environ 409:3564 - 3577

[14] Vazquez-Roig P, Blasco C, Picó Y (2013) Trends Anal Chem 50:65 - 77

[15] Boleda MR, Huerta-Fontela M, Ventura F, Galceran MT (2011) Chemosphere 84:1601 - 1607

[16] Pedrouzo M, Borrull F, Pocurull E, Marcé RM (2011) J Sep Sci 34:1091 - 1101

[17] Langford KH, Reid M, Thomas KV (2011) J Environ Monit 13:2284 - 2291

[18] European Commission Decision 2002/657/EC (2002) Off J Eur Commun 221:8-36

[19] Pozo OJ, Sancho JV, Ibáñez M, Hernández F, Niessen WMA (2006) Trends Anal Chem 25:1030 - 1042

[20] Hernandez F, Sancho JV, Pozo OJ (2005) Anal Bioanal Chem 382:934 - 946

[21] Lehotay SJ, Mastovska K, Lightfield AR, Gates RA (2010) J AOAC Int 93:355 - 367

[22] Stahnke H, Kittlaus S, Kempe G, Alder L (2012) Anal Chem 84:1474 - 1482

[23] Martínez Bueno MJ, Uclés S, Hernando MD, Fernández-Alba AR (2011) Talanta 85:157 - 166

[24] Bijlsma L, Sancho JV, Pitarch E, Ibáñez M, Hernández F (2009) J Chromatogr A 1216:3078 - 3089

[25] Gracia-Lor E, Sancho JV, Hernández F (2011) J Chromatogr A 1218:2264 - 2275

[26] Hernández F, Bijlsma L, Sancho JV, Díaz R, Ibáñez M (2011) Anal Chim Acta 684:96 - 106

[27] Ort C, van Nuijs ALN, Berset J-D, Bijlsma L, Castiglioni S, Covaci A, de Voogt P, Emke E, Fatta-Kassinos D, Griffiths P, Hernández F, González-Mariño I, Grabic R, Kasprzyk-Hordern B, Mastroianni N, Meierjohann A, Nefau T, Ostman M, Pico Y, Reid M, Slobodnik J, Terzic S, Thomaidis N, Thomas KV (2014) Addiction (accepted)

2.3 Discussion of the results

Compound selection

In 2007, the University Jaume I – Fundació Bancaixa approved a project proposal applied by Prof. Dr. Félix Hernández (Ref. P1 1B2007-13) "*Potencial de la cromatografía líquida acoplada a espectrometría de masas (QqQ, TOF) como herramienta analítica para la estimación del consumo poblacional e individual de estimulantes y drogas de diseño*". Its main objective was to study the potential of liquid chromatography coupled to tandem mass spectrometry, both QqQ as QTOF, for the determination of IDs in urban wastewaters. The project initially pretended to focus on widely consumed drugs in the province of Castellón and those drugs which were of high interest for local authorities. Information was gathered from the Unidad de Conductas Adictivas (Unit of Addicitive Behaviour), the Comisaría de la Policia Nacional (National Police), the Guardia Civil (Spanish gendarmerie), and also from different Public Health services centered in Castellón. Once the most relevant IDs were selected, a detailed literature search was performed in order to obtain more information regarding their activity, toxicity, structure, physical / chemical properties, etc as well as metabolism. The latter is important as in some cases the presence of IDs are studied through the presence of their metabolite(s) *i.e.* drug biomarkers, such as the case with 11-nor-9-carboxy-Δ^9-THC (THC-COOH), the metabolite of cannabis [Castiglioni *et al.* 2008]. In addition, a literature search regarding IDs detected in wastewater was performed. At that time, the first applications of LC-MS/MS methods to the simultaneous analysis of IDs in urban wastewater were recently published [Castiglioni *et al.* 2006; Boleda *et al.* 2007; Bones *et al.* 2007; Huerta-Fontela *et al.* 2007]. Although information was scarce and incomplete, a pre-selection of IDs could be made and in the first study, amphetamines and ATS, cocaine and its metabolites, and THC-COOH were included. In the second study, performed three years later, this list was extended by opioids (heroin, morphine, 6-MAM (exclusive metabolite of heroin) and codeine) and ketamine. Accordingly, the developed analytical method allowed the simultaneous quantification and confirmation of amphetamines, MDMA, cocaine, cannabis, opioids and ketamine in a single run. Probably due to the different physico-chemical characteristics of these IDs,

most of the reported LC-MS/MS methods analysed only some of the selected ID groups. It is important to mention that some metabolites of amphetamines and cocaine were not included since standards were out of stock, but more importantly these compounds (MDA, MDEA, Cocaethylene, norBenzoylecgonine and norCocaine) were hardly detected in waters.

MS/MS Optimization

Full scan spectra and MS/MS compound- and instrument-dependent parameters (e.g. cone and collision voltages) for SRM determination of each analyte are the first parameters to be optimized. Ionization of the selected IDs and their metabolites was performed by means of ESI under positive ionization mode, including THC-COOH. In all cases the protonated molecule $[M+H]^+$ was selected as precursor ion, for both quantification and confirmation transitions. The only exception was the confirmation of amphetamine in the second study, where, the transition (119 > 91) promoted by in-source fragmentation was selected to have additional confirmation, due to the poor sensitivity of the confirmatory transition based on the protonated molecule as precursor ion (136 > 65).

In general, for quantification (Q) the most sensitive transition was selected to favour the quantification at low concentrations. In addition, two transitions were acquired for confirmation (q). At this point, it was also important to know fragmentation of analytes and to select specific fragments for both quantification and confirmation. Non-specific losses, such as water, carbon dioxide or formic acid, were avoided if possible, as they are more prone to be interfered in complex matrices.

Sensitivity improvement

The major differences between *scientific article 1 and 2* were the change towards a more generic SPE cartridge and the use of state-of-the-art UHPLC-MS/MS with triple quadrupole (TQS) mass analyzer. The TQS mass analyzer is equipped with the "step-wave" technology, which is based on a stacked ring ion guide (**Fig. 2.1**). This Step-wave device, in theory, maximizes ion transmission from the source to the mass analyser and allows for the active removal of neutral contaminants, providing an enhancement to

the overall signal-to-noise ratio. It ensures that methods remain robust for longer time, even with complex matrices [http://www.waters.com/waters/en_US/StepWave/nav.htm?cid=134673601]. The same technology is applied in the collision cell.

Figure 2.1. Step-wave device [http://www.waters.com/waters/en_US/StepWave]

Table 2.1 shows the comparison of instrumental LODs of the TQD and TQS mass analyzers for various compounds analyzed in both studies. Improved sensitivity was observed for most compounds, but in particular for THC-COOH, when using the TQS mass analyzer with the stepwave technology. However, amphetamine showed a decrease in sensitivity for all transitions monitored.

Table 2.1. Comparison of instrumental LODs of the TQD and TQS triple quadrupole mass analyzers

Compound	LOD (fg), TQD analyzer	LOD (fg), TQS analyzer
Amphetamine	570	950
Methamphetamine	300	200
MDMA	250	130
Cocaine	260	130
BE	180	110
THC-COOH	6700	600

The change made in SPE was in principle beneficial from the point of view of a wider application range. However, the use of a more generic HLB cartridge might lead to an increase of matrix components that remained retained in the cartridge and might therefore have resulted in more ion suppression and consequently lower sensitivity of amphetamine, something that is explicitly mentioned and discussed in *scientific article 2*.

UHPLC optimization

The mobile phase composition can be relevant to improve LC separation and enhance the detector response [Niessen *et al.* 2006]. Well defined chromatographic peaks are essential for accurate peak integration and subsequent compound quantification. Furthermore, an efficient separation is valuable to avoid or minimize matrix effects. The importance of good chromatographic separation is also clearly illustrated in ***figure 3 of scientific article 1***, where two metabolites of cocaine (BE and norcocaine), shared the same SRM transition (290.1 > 168.2), and co-elution needed to be avoided. This became more important since the corresponding transition was used for BE quantification; thus if co-elution would have occurred the concentration of BE and consequently the consumption of cocaine could have been overestimated. Hence, the relevance of appropriate chromatographic separation and composition of the mobile phases were important issues in this work. Mobile phases based on organic solvent (acetonitrile and methanol) and water or buffer solutions of ammonium acetate and ammonium formate were tested. Methanol was selected as it showed better response compared to acetonitrile. The addition of ammonium acetate to the water phase allowed improving peak shapes, resolution and efficiencies for several target compounds especially when analyzing wastewater samples. This could be further optimized by varying mobile phase ionic strength *i.e.* the buffer concentration (1 - 30 mM). An ammonium acetate concentration of 5 mM was finally selected.

The effect of pH by the addition of formic acid to both the aqueous and organic phase was also evaluated. Sensitivity and chromatographic performance improved for all compounds, except for THC-COOH. Therefore, formic acid was not added to MeOH so that its effect on this relative late-eluting compound was diminished. The optimum

amount/percentage of formic acid added, to favour the formation of the protonated molecule, varied from instrument to instrument. While 0.1% of formic acid provided the best results using the TQD mass analyzer (*scientific article 1*), 0.01% formic acid resulted in the highest sensitivity using the TQS mass analyzer (*scientific article 2*).

Sample treatment and matrix effects

The extent of sample preparation can determine the chromatographic/ analytical performance, and also the achievement of the required selectivity and sensitivity in the determination of IDs at very low (sub-ppb) concentrations. In the works presented in this chapter, off-line SPE was applied preceded by either centrifugation (*scientific article 1*) or filtration (*scientific article 2*) to prevent SPE from clogging. It is worthwhile to mention that no losses were observed using this filter. Two different cartridges were considered regarding the SPE step: Oasis HLB and Oasis MCX. Oasis HLB is able to retain a large variety of analytes of different polarities, whereas Oasis MCX allows improved selectivity towards basic analytes.

In both studies, extraction efficiency, matrix effects and process efficiency were estimated performing preliminary experiments with three different sets of samples, according to the procedure described by Matuszewski *et al.* 2003. Set #1 consisted of a mixed standard solution in solvent, which included all corresponding ILIS. Set #2 consisted of water samples, which were first extracted using the SPE procedures described in *scientific article 1 and 2*, and the eluates were then spiked with the mixed standard and ILIS solutions. In set #3, water samples were first spiked at the same concentrations as sets #1 and #2, and then SPE extracted as described. Extraction efficiency (i.e. absolute recovery) was calculated by comparing the peak areas of set #2 to set #3; matrix effects by comparing set # 2 to set #1; and total process efficiency (i.e. relative recovery) by comparing set #3 to set #1. By performing these experiments, matrix effects, absolute recoveries and relative recoveries were evaluated in order to improve and optimize the overall analytical methodology.

The extraction efficiency of both types of cartridges was estimated for the selected analytes and showed satisfactory absolute recoveries (70 - 120%). In the first study, the

extraction by Oasis MCX was optimized to take advantage of the improved selectivity towards the target basic IDs. In the second work, the interest of performing additional wide-scope screening of other potential contaminants by UHPLC-QTOF MS (Hernández *et al.* 2011 – *scientific article 3*), induced a change to the more generic Oasis HLB. Yet for amphetamine an additional clean-up using Oasis MCX cartridges and dispersive primary secondary amine (PSA) was needed as thoroughly explained in *scientific article 2*.

As mentioned in the general introduction, matrix effects are one of the principal difficulties when developing analytical methods based on LC-MS/MS. It is getting even more complicated in multi-residue analysis as matrix effects are instrument, matrix and certainly also compound dependent. There are various approaches to deal with matrix effects in quantitative analysis. In the scientific articles presented in this chapter, special effort was made to reduce matrix effects and improve analytical performance without a substantial loss of sensitivity. Sample treatment based on SPE, as described above, sample dilution, and/or decrease in sample injection volume were applied in order to improve method performance and sensitivity. Normally, complete elimination of matrix effects is not feasible, especially in complex environmental and wastewater samples. Thus, besides that those effects should be minimized, a compensation/correction for matrix effects is needed for accurate quantification.

The addition of appropriate internal standards as surrogate *i.e.* prior to any sample manipulation, is one of the best approaches to compensate not only for matrix effects but also for potential errors associated to sample preparation. In that respect, the own labelled analyte is considered to be an ideal internal standard, as it shows (almost) identical behaviour to the target analyte in sample pre-treatment, chromatography and ionization. Fortunately, in both studies, deuterated labelled standards were available for each compound, except for norcocaine and norbenzoylecgonine (*scientific article 1)*. In these cases, as analyte own ILIS were not commercially available, alternative ILIS (cocaine-d_3 and benzoylecgonine-d_3, respectively) were tested.

All compounds suffered matrix ionization suppression effects, but these effects could be compensated by their corresponding ILIS. Cocaine-d_3 was found to be a good alternative for correct quantification of norcocaine. Norbenzoylecgonine was the only exception, and showed ionization enhancement in contrast to norcocaine, cocaine-d_3, and the rest of internal standards (e.g. benzoylecgonine-d_3), which showed matrix ionization suppression (**Fig. 2.2, Table 2.2**). Under these circumstances, the closest ILIS benzoylecgonine-d_3 was not able to compensate matrix effects for norbenzoylecgonine. Therefore, norbenzoylecgonine was finally quantified without using any internal standard.

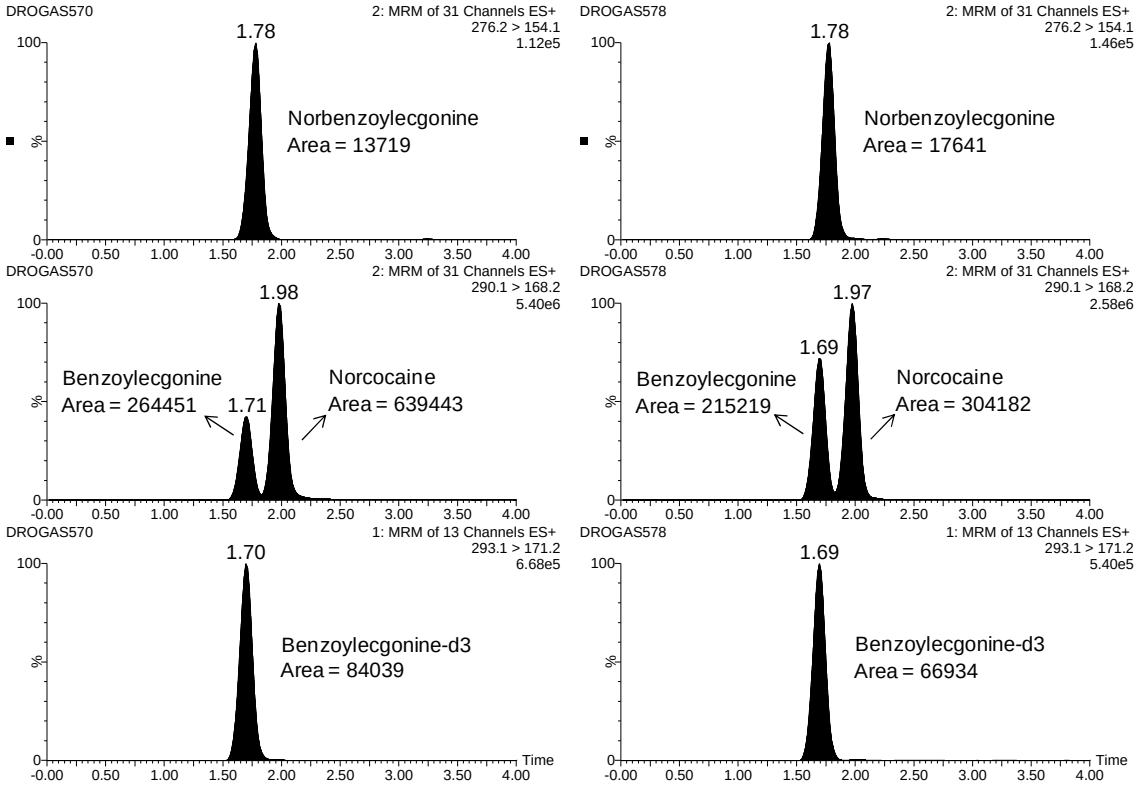


Figure 2.2. SRM chromatograms of norbenzoylecgonine, benzoylecgonine and benzoylecgonine-d_3 in solvent (left) and surface water (right).

Table 2.2. Peak areas, in solvent and surface water, for norbenzoylecgonine, benzoylecgonine and the deuterated compound benzoylecgonine-d_3.

	Area in solvent		Area in matrix
Norbenzoylecgonine	**13719**	**<**	**17641**
Benzoylecgonine	**264451**	**>**	**215219**
Benzoylecgonine-*d3*	**84039**	**>**	**66934**

Method validation

The performance of the method was evaluated in terms of linearity, limits of quantification, limits of detection, accuracy and precision, for each matrix studied. Data for method validation can be found in ***tables 2, 3 and 4 of scientific article 1***, and ***tables 2 and 3 of scientific article 2***.

Representative matrix-matched standards could not be easily prepared, due to the presence of some IDs in water samples, especially influent wastewaters, and because of the variations in sample composition from one sample to the other. Therefore, calibration was made in solvent, on the basis that matrix effects were compensated by the use of appropriate ILIS. *Linearity* was studied by analyzing in triplicate standard solutions in solvent at six concentration levels from 0.5 to 25 µg/L. The exceptions were amphetamines and ATS (2 to 70 µg/L), and THC-COOH (20 to 600 µg/L), when performing analysis using the TQD mass analyzer. This was related to the sensitivity reached for these analytes. With few exceptions, the correlation coefficients were greater than 0.99.

The estimation of realistic *limits of detection* (LODs) and *limits of quantification* (LOQs) is complicated in sewage water, where notable variations between samples commonly occur and where true blank samples are not feasible to be obtained for some analytes (e.g. benzoylecgonine). The LOQ objective was taken as the lowest concentration level for which the method was fully validated using spiked "blank" samples with satisfactory recovery and precision (*scientific article 1*). With this approach, the LOQs obtained are

fully supported by application of the overall method at this concentration. Although this procedure is surely the most realistic and most supported by laboratory data, the inconvenience is that these LOQs are much higher than those reported in the scientific literature, which are commonly based on statistical estimation. Thus, it may seem that the method is much less sensitive than others, while it is not. Hence, in *scientific article 2,* the LOQs were estimated in agreement with most papers from the literature, *i.e.* for a S/N ratio = 10 using the quantification transition, from chromatograms of "blank" samples spiked at the lowest validation level tested (taking into account the analyte concentration found in the "blank", in those cases where the sample was positive for a given ID). In addition, the presence of a peak for at least one of the confirmation transitions with S/N ratio ≥ 3 was required. Thus, a possible positive finding at the LOQ level could be quantified but also confirmed.

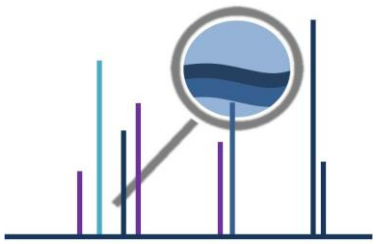
The *accuracy* and *precision* of the method were estimated by means of recovery experiments, using "blank" wastewater samples spiked at two concentration levels (LOQ and 10LOQ). Obviously, samples needed to be previously analyzed, since genuine blank sample were not available. Concentrations of target compounds occasionally found in these "blank" samples were subtracted from the spiked samples. All experiments were carried out in quintuplicate at each level and the results obtained for most compounds were satisfactory, with recoveries between 70 – 120% and precision (RSD) below 20%. (Data shown in ***tables 2, 3 and 4, scientific article 1***; ***tables 2 and 3, scientific article 2***).

Application to water samples

The developed analytical methodologies described in this chapter were applied to the determination of IDs and their metabolites in real water samples. The results of these studies are presented in chapter 5 "Occurrence of illicit drugs in the aquatic environment" and in chapter 6 "European-wide collaboration". In general, the illicit drug most frequently and abundantly found in wastewater samples of Castellón was cocaine; both as parent compound and as its main metabolite BE. Concentrations of cocaine and BE on weekdays were lower than during weekends, which suggest that this drug is increasedly consumed in the weekends. Other drugs detected in

wastewater of Castellón were cannabis (by means of THC-COOH), MDMA, codeine and morphine. The latter two were probably found due to medical use rather than illicit use. An interesting set of samples were taken during a well-known international music event (Festival Internacional de Benicàssim, FIB), which highlighted the presence of high levels of MDMA, amphetamine, MDA and the cocaine metabolites, norbenzoylecgonine and cocaethylene. (Data shown in ***table 5, scientific article 1***; ***figure 1, scientific article 8***).

Confirmation of positive findings was carried out according to the guidelines often applied in environmental analytical chemistry. The acquisition of two SRM transitions per compound is considered satisfactory for a reliable confirmation of the presence of a compound [2002/657/EC; SANCO/12495/2011]. However, in both studies presented in this chapter, three transitions per compound were acquired allowing even more confidence to the data produced. Furthermore, compliance of at least one ion ratio between the recorded transitions (Q/q_1 and/or Q/q_2) and retention time, within the maximum allowed deviations in relation to reference standards, is required before reporting a sample as positive. In our articles, these confirmation parameters were discussed with graphical evidences supporting the confirmation issue (***figure 5, scientific article 1***). This is not common in the literature, where these critical parameters are often not discussed or unreported, despite that reliable confirmation is of outstanding relevance and reporting false positives or false negatives has to be avoided. In this particular field, data reported on IDs consumption through wastewater analysis receives generally much attention of the media (press, radio, television) and has high impact on the community. Therefore, the analytical results should be carefully presented and reported.



CHAPTER 3

Screening by High Resolution Mass Spectromety

3.1 Introduction

LC-MS/MS is nowadays the preferred technique for the quantification of small molecules in environmental sciences and in some other fields (e.g. food safety, forensic sciences). The analytical methodologies described in chapter 2, using low resolution triple quadrupole instruments, demonstrated excellent sensitivity, selectivity, accuracy, precision and reproducibility, for trace and ultra-trace analysis of IDs in wastewater and surface water. However, these water samples may contain hundreds if not thousands of substances that are in daily use in households and industry at varying concentrations forming a major source of contamination. This fact discloses an important limitation of LC-MS/MS, where the number of compounds to be included in the method is restricted. Thus, a multitude of organic contaminants, including other IDs, not initially considered in the scope of the method, cannot be revealed, even if they are present at high levels in the sample. The evolution of high resolution, high accuracy mass spectrometry (HRMS), coupled with liquid chromatography, and improvements in extraction, enrichment and analytical procedures has opened up new opportunities for the detection of polar organic contaminants in complex water samples. HRMS offers a series of advantages against other types of analyzers, such as triple quadrupole mass analyzers. Some of these advantages but also limitations are outlined in this chapter.

HRMS is a very attractive analytical technique for multi-component screening and identification as it provides the required selectivity and sensitivity by combining full spectrum MS data with high mass resolution that makes it able to measure accurately the mass of any ionizable analyte in the sample. In theory, the presence of an unlimited number of compounds can be investigated in a single experiment, without being dependent on the pre-selection of analytes or having reference standards available. The power of accurate mass is found in the determination of the elemental composition of a compound or its fragment ions. The increased resolution of HRMS permits to measure accurately the mass of a compound in complex matrices and therefore also discriminates between compounds with similar mass. Hence, isobaric compounds, which have the same nominal mass (*i.e.* at one decimal), but different elemental composition and thus different exact mass, can be distinguished by accurate mass measurements.

On the contrary, when using a low resolution instrument, isobaric compounds can be discriminated only from their retention time and/or fragmentation. But when chromatographic separation is not fully achieved, a false positive or negative might be reported owing to a lack of specific fragments or due to noncompliance of ion ratios [Pozo *et al.* 2006]. HRMS allows discriminating isobaric compounds from differences in mass defect reducing the possibilities of false identification. Moreover, an elevated mass resolution allows the extraction of ion chromatograms (XIC) at narrow mass windows (normally, 10 – 20 mDa) for a selected compound. A narrow window allows a reduction of isobaric interferences and consequently improves selectivity (Hogenboom *et al.* 1999; Petrovic *et al.* 2006; Sancho *et al.* 2006; Ibáñez *et al.* 2008).

The resolving power of an instrument is inextricably linked to the achievable mass accuracy. An illustrative example is presented in **Figure 3.1**, where a resolution of 15.000 FWHM did not allow the detection of the herbicide Sulcotrione. When ion peaks are not fully resolved, the resulting measured mass profile will be the sum of the individual mass profiles and the top of the combined profile will be somewhere in between the exact masses of the individual peaks. As a consequence, the mass assignment, which is based on a centroiding algorithm of the detected profile, will result in an incorrect mass

for the analyte, and, as a consequence, the analyte might not be detected in target analysis, depending on the mass window selected [Peters *et al.* 2010]. However, when performing analysis at higher resolution i.e. 50.000 FWHM, Sulcotrione could be resolved from co-eluting interferences (*m/z* 329.0067 vs. *m/z* 329.02475) and the accurate mass led to its correct identification.

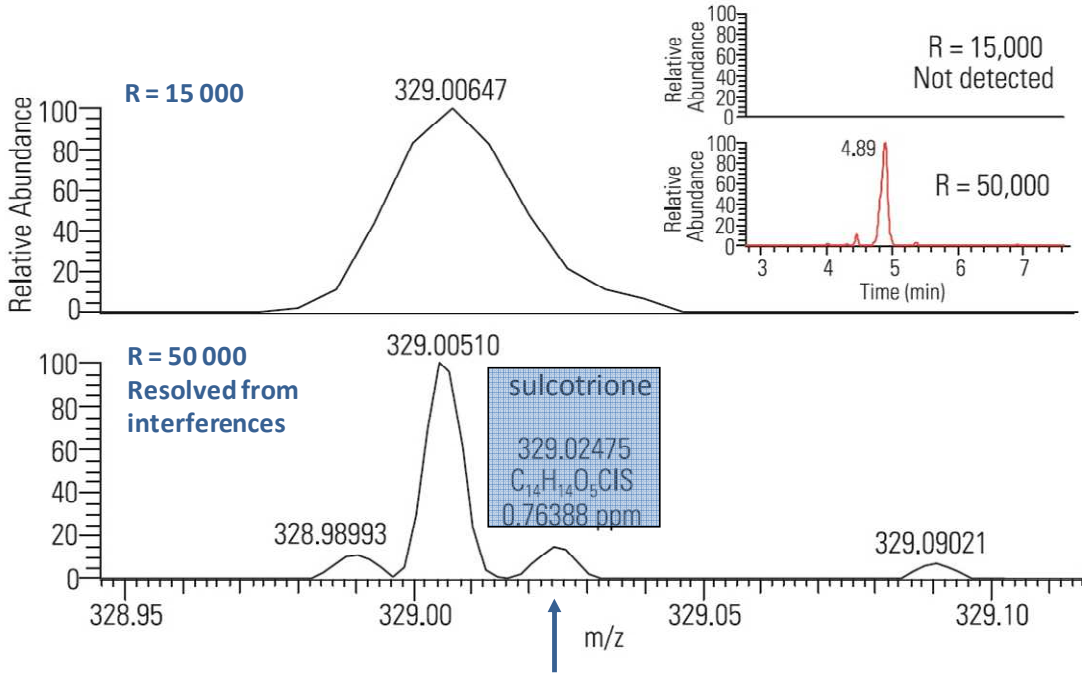


Figure 3.1: Detection of sulcotrione by applying an elevated mass resolution (example of Thermo Scientific).

The high quality of data obtained by HRMS allows the determination of the elemental composition of a compound with high confidence. Subsequently, an interesting feature of HRMS in the identification process is the application of isotopic filters. The presence of a characteristic isotopic pattern of atoms such as carbon, chlorine, bromine or sulfur in the molecule helps to reduce the number of possible elemental compositions or to confirm the presence of a compound in the sample.

HRMS, thanks to the full-scan accurate-mass acquisition, provides a notable amount of chemical information with high sensitivity and selectivity. For the screening of multiple compounds, three approaches can be applied [Hernandez *et al.* 2005b; Krauss *et al.* 2010]:

(i) *Target analysis* – where reference standards are necessary to determine the concentration in the sample and to match the measured retention time (RT) and, if available, tandem mass spectrum (MS/MS).

(ii) *Suspect (Post-target) screening* – where compounds, even not of interest at the time of sample analysis, are selected after full-spectrum acquisition. No reference standards are necessary in a first step, as searching mainly relies on information (chemical formula, accurate mass and isotopic pattern) available for the precursor ion. Subsequently, information on the presence of fragment ions may allow the tentative identification of the suspect compound, which needs to be finally confirmed by injection of reference standards

(iii) *Non-target screening* – where compounds eluting from the chromatographic column may be detected and subsequently identified without any kind of previous selection. Here, the analyst is searching for actual unknown compounds, as no previous information about the compounds to be investigated is taken into account.

The ability to screen for hundreds of organic contaminants in targeted approaches, even after measurement (post-target / retrospective), and the possibility to detect unknown non-target chemicals has increased the interest of using HRMS in environmental sciences [Hogenboom *et al.* 2009; Krauss *et al.* 2010; Nurmi *et al.* 2011; Hernandez et al 2012; Hug *et al.* 2014; Schymanski *et al.* 2014].

The HRMS instruments most widely used for screening purposes of environmental samples are TOF and Orbitrap. Both instruments provide high mass resolving power and high mass accuracy, but each analyzer has its specific strengths and drawbacks. The choice of instrument depends mainly on the application, adaptability, and costs, but

obviously also on the availability within the laboratory. Most publications so far use TOF MS instruments with mass accuracies typically less than 5 ppm and mass resolving power of 20,000 FWHM. It combines full spectrum sensitivity and elevated acquisition speed, which makes it compatible with UHPLC. The Fourier-transform (FT) Orbitrap MS technology, on the other hand, is being increasingly applied due to the combination of very high resolving power (up to100,000 FWHM), mass accuracy (2 - 5 ppm), and linear dynamic range.

The development of hybrid instruments i.e. the combination of two different mass analyzers, has revolutionized the application of TOF and Orbitrap. Hybrid instruments, such as QTOF MS and LTQ Orbitrap MS, offer more possibilities in screening and identification, based on high resolution accurate mass measurement of precursor and/or product ions. By performing MS/MS experiments, the recording of full-scan accurate-mass product ion spectra is feasible, yielding relevant information for compound confirmation or structure elucidation. However, the potential of hybrid systems running in MS/MS mode is limited as it implies the pre-selection of analyte precursor ions. An alternative approach, feasible with the QTOF system used in this thesis, is the MS^E acquisition strategy [Castro-Perez *et al.* 2002; Plumb *et al.* 2006; Weaver *et al.* 2007; Tiller *et al.* 2008], which allows collecting simultaneously information on both (de)protonated molecules and fragment ions, by acquiring data at low and high collision energy in a single injection, without the need of selecting a precursor ion. Furthermore, practical parameters, such as isotopic pattern and double bond equivalent (DBE), can be used to facilitate a more reliable identification [Hernandez *et al.* 2011 – *scientific paper 3*]. As for the hybrid LTQ Orbitrap MS, accurate mass measurements are combined with the trapping capacity and MS^n scan function of the linear ion trap. The MS can operate in a data-dependent-acquisition (DDA) mode in which both MS and MS^n spectra can be acquired without the need to specify precursor masses. However, an inclusion lists can be also used to obtain MS/MS data of target compounds [Bijlsma *et al.* 2013 – *scientific paper 5*].

As outlined above, LC-HRMS(/MS) enable new screening strategies, which have allowed the detection and identification of various IDs in environmental and

wastewater samples with high confidence due to the high mass accuracy measurements. However, these new approaches also bring new challenges. LC-HRMS(/MS) data has a wealth of information and the number of analytes that can be detected is too large for the traditional visual inspection of extracted ion chromatograms (XICs). The gathering of the screened *m/z* for identification and confirmation remains very time-consuming. Therefore, in a post-target approach, extraction of the analytes of interest from the raw data has to be done automatically by software algorithms provided by manufacturers, to facilitate this process. To this aim, databases with the target analytes are needed. Once a database has been established, software parameters for analyte detection need to be optimized in order to obtain a fit-for-purpose balance between false positives and false negatives reported by the software. Such parameters may include retention time tolerances, accurate mass tolerances, and requirements for presence of multiple adducts, isotope fits, fragment ions, ion ratios and response thresholds [Mol *et al.* 2012].

This chapter focuses on HRMS detection, a sophisticated HRMS approach that however cannot detect any analyte if the preceding analytical steps (extraction, enrichment/purification, LC separation, ionization) are not suitable [Krauss *et al.* 2010]. Therefore, in order to perform a wide-screening, not selective sample treatments and generic chromatographic separations are required to broaden the system applicability to as many compounds as possible.

3.2 Scientific articles

The increasing interest of using HRMS(/MS) instruments in environmental sciences mainly relies on the high quality of the analytical data obtained and on the capability to screen for a large number of compounds in a single experiment. After sample analysis and data acquisition, all information obtained can be processed and the presence of newly identified compounds can be investigated at any time in previously analyzed samples, simply by reprocessing the data. In this chapter, both QTOF MS and LTQ Orbitrap MS were employed for the screening of IDs in complex water samples, demonstrating the strong potential of these techniques.

In the first two studies (*scientific article 3* and *scientific article 4*), the potential of QTOF MS combined with UHPLC for screening and confirmation of drugs of abuse, prescription drugs with potential of abuse, and their metabolites in wastewater samples is illustrated. Analytes were concentrated using mixed-mode Oasis MCX cartridges, which previously demonstrated satisfactory results for several IDs [Bijlsma *et al.* 2009 – *scientific article 1*]. High quality accurate mass data of both protonated molecules and fragment ions could be obtained in a single run, with the QTOF instrument operating under MS^E acquisition mode. A database including the exact masses and empirical formulae of each compound was established, and together with powerful software (ChromaLynx XS) provided by Waters, a relative rapid and simple reviewing of the data was feasible. The combination of all information provided (*e.g.* accurate mass, isotopic distribution, DBE) allowed the tentative identification of several IDs in complex wastewater samples, even without using reference standards. UHPLC-QTOF MS was proven to be a powerful and efficient technique for rapid wide-scope screening and identification of IDs in complex aquatic matrices.

The third study (*scientific article 5*) presents the development, validation and application of an analytical methodology based on full-spectrum accurate-mass and MS/MS acquisition provided by LC-LTQ FT Orbitrap MS, for qualitative and quantitative analysis of 24 IDs and relevant metabolites in sewage water. The potential of HRMS in qualitative analysis is widely recognized, however quantitative analysis has not been explored much. Therefore, an objective within this study was to demonstrate the

quantitative capabilities of Orbitrap MS for IDs in complex wastewater samples. The developed methodology consisted of automatic SPE using Oasis HLB cartridges to concentrate targeted drugs. ILIS were added to the samples as surrogates, for matrix effects correction and possible losses during sample treatment. Chromatographic separation was achieved within 42 minutes, with an XBridge C18 column (150 mm x 2.1 mm I.D., particle size 3.5 μm) under HPLC optimized conditions. Full-scan accurate-mass data of the protonated molecules were acquired and simultaneous MS^n measurements were made to obtain information of fragment ions generated in the linear ion trap. The methodology was satisfactory validated, and in addition to the excellent qualitative capabilities, Orbitrap provided good quantification performances at sub-ppb analyte levels. Finally, Orbitrap also allowed retrospective investigation for post-targeted compounds, such as the ketamine metabolites norketamine and dehydronorketamine. As stated in the general introduction (Chapter 1), *scientific article 5* results from a close collaboration between the University Jaume I and KWR Watercycle Research Institute, where the PhD candidate performed a two-months stay to work on Orbitrap technology. The contribution of the PhD candidate to this manuscript is explained in Chapter 1, section 1.4.

The results of the three studies presented in this chapter have been published in:

- *Analytica Chimica Acta* 684, **2011**, 96 – 106
- *Illicit Drugs in the Environment: Occurrence, Analysis, and Fate Using Mass Spectrometry, Castiglioni S, Zuccato E, (Eds.). Wiley, Hoboken NJ (USA), ISBN: 978-0-470-52954-6 Chapter 4,* ***2011****, 69 – 85*
- *Analytica Chimica Acta* 768, **2013**, 102 – 110

Chapter 3.2.1, scientific article 3

Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography–quadrupole-time-of-flight-mass spectrometry

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Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography–quadrupole-time-of-flight-mass spectrometry

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Abstract

This work illustrates the potential of hybrid quadrupole-time-of-flight mass spectrometry (QTOF MS) coupled to ultrahigh pressure liquid chromatography (UHPLC) to investigate the presence of drugs of abuse in wastewater. After solid-phase extraction with Oasis MCX cartridges, seventy-six illicit drugs, prescription drugs with potential for abuse, and metabolites were investigated in the samples by TOF MS using electrospray interface under positive ionization mode, with MS data acquired over an *m/z* range of 50–1000 Da. For 11 compounds, reference standards were available, and experimental data (e.g., retention time and fragmentation data) could be obtained, facilitating a more confident identification. The use of a QTOF instrument enabled the simultaneous application of two acquisition functions with different collision energies: a low energy (LE) function, where none or poor fragmentation took place, and a high energy (HE) function, where fragmentation in the collision cell was promoted. This approach, known as MS^E, enabled the simultaneous acquisition of full-spectrum accurate mass data of both protonated molecules and fragment ions in a single injection, providing relevant information that facilitates the rapid detection and reliable identification of these emerging contaminants in the sample matrices analyzed. In addition, isomeric compounds, like the opiates, morphine and norcodeine, could be discriminated by their specific fragments observed in HE TOF MS spectra, without the need of reference standards. UHPLC–QTOF MS was proven to be a powerful and efficient technique for rapid wide-scope screening and identification of many relevant drugs in complex matrices, such as influent and effluent urban wastewater.

Keywords

Illicit drugs of abuse, ultra-high-pressure liquid chromatography, time-of-flight mass spectrometry, MS^E, wide-scope screening, urban wastewater

1. Introduction

In last few years, there is increasing concern over the presence of emerging contaminants in aquatic ecosystems [1, 2]. Similar to other emerging pollutants, like pharmaceuticals, personal care products, or pesticide metabolites, illicit drugs have received much attention since their presence was reported in surface water and urban wastewater [3 - 5].

Several multi-residue LC–MS/MS methods have been developed for the simultaneous quantification and identification of illicit drugs in environmental samples [6, 7] and have been applied in water monitoring studies [8, 9]. The information obtained from these studies can help in understanding the potential impact of these compounds in the environment. Furthermore, new strategies have been reported for multi-target screening of drugs in forensic samples [10] and in environmental samples [11] using liquid chromatography (LC) coupled to a hybrid quadrupole linear ion trap mass spectrometer (QqLIT) or a hybrid linear ion trap Orbitrap (LTQ-Orbitrap) mass spectrometer, respectively. By the latter approach, several illicit drugs and metabolites were positively identified, based on accurate mass and retention time, in surface water and effluent wastewater. The LTQ-Orbitrap configuration has also been successfully applied to identification of transformation products of organic contaminants in natural waters [12].

Most of the methods developed until now for research of illicit drugs in the environment are based on LC–MS/MS using a triple quadrupole (QqQ) analyzer, where the analytes are pre-selected before MS-data acquisition. The excellent sensitivity and selectivity achieved in selective reaction monitoring (SRM) mode makes this technique very attractive for quantification and identification of compounds detected in samples [5]. Recent papers have also been reported using ultrahigh pressure liquid chromatography (UHPLC) coupled to triple quadrupole mass spectrometry for rapid separations with improved chromatographic resolution. This combination is ideal for trace analysis in complex environmental samples, including the determination of drugs of abuse in wastewater [13 - 16]. In spite of the strong potential of this approach, there are some disadvantages. Due to dwell times and time segment restrictions, only a limited number

of ions may be selected. Moreover, reference standards are required for each target compound for SRM optimization. Most of the illicit drug standards are expensive, and it is often time-consuming to acquire them, due to the strict regulations applied to these substances. Another important drawback is that other potential contaminants not included in the method are generally not detected, even if they are present at high levels in the sample. This occurs in "pre-target" approaches, where only pre-selected *m/z* ions are monitored.

On the contrary, TOF instruments offer the possibility to investigate the presence of compounds once the analysis has been performed and data acquired, without being dependent on the pre-selection of analytes. This is possible due to full-scan acquisition at good sensitivity with TOF instruments. The benefit of this "post-target" approach, where the selection of compounds to be searched is made after the MS acquisition, is the possibility to detect a large number of contaminants, without using reference standards or performing additional analyses. The sensitive full spectrum MS data and the high mass resolution and mass accuracy provided by TOF-MS makes this technique especially suited for wide-scope screening. TOF-MS has some interesting features that facilitate a reliable identification. Extracted ion chromatograms (XIC) at selected *m/z* can be performed from full-scan data at accurate mass with high sensitivity, selecting narrow *m/z* windows (e.g., ±0.02 Da). In addition, the narrow mass window leads to a reduction of interferences from isobaric compounds that might be present in samples and to an increase in signal-to-noise [17 - 21]. In addition, isotopic filters, such as carbon, chlorine, bromine, or sulfur filters can be applied. The presence of a characteristic isotopic pattern further limits the number of possible elemental compositions for a certain accurate mass and helps to confirm the presence of a given compound in the sample. The match between empirical and theoretical data is given by parameters like isotope fit (i-FIT) or SigmaFIT, depending on the manufacturer. These values are calculated, taking into account not only the isotopic distribution, but also the accurate mass [20, 22].

LC–TOF-MS has shown a strong potential for screening and confirmation in environmental samples. Compounds like pesticides, pharmaceuticals, and antibiotics

have been successfully analyzed [18 - 21]. In addition, several studies have screened compounds of toxicological interest (including illicit drugs) in forensic samples [22 - 24].

Pesticide residues have been one of the most investigated environmental contaminant by LC–TOF-MS, although only in the last few years. However, wide-scope screening, including hundreds of compounds (300–400 analytes) has only been applied in a few cases [25 - 27]. It is expected that LC–TOF-MS is also an attractive technique for wide-scope screening of illicit drugs in environmental samples using a similar post-target approach. Due to their medium polarity, most drugs of abuse and their metabolites are more suited to LC analysis rather than GC analysis.

Hybrid quadrupole-TOF (QTOF) MS analyzers can work as a single TOF, but they can also provide relevant structural information by obtaining full-scan spectra of products ions at accurate mass. QTOF-MS/MS experiments are highly useful for elucidating the structures of unknown compounds and are an excellent way to confirm potential positives revealed by TOF-MS or QqQ analysis. However, the pre-selection of analyte precursor ions is required in the quadrupole for MS/MS product ion generation, and typically, isotopic pattern information is lost.

With MS^E experiments, both precursor molecule and fragment ion data are enabled in a single acquisition, without the need of selecting a precursor ion. Using this approach, the QTOF instrument is used in TOF mode, promoting fragmentation in the collision cell. MS^E experiments involve the simultaneous acquisition of exact mass data at low and high collision energy [28 - 32]. By applying low energy (LE) in the collision cell, fragmentation is minimized, and the information obtained corresponds normally to non-fragmented ions, related to the parent molecule. However, at high collision energy (HE), fragmentation will take place, resulting in abundant fragments. In some cases, the (de)protonated precursor molecule can also be observed in HE TOF-MS spectra. Although these ions are not generated in a traditional MS/MS manner, analogous terms are sometimes used, due to similarities in both approaches when using the same QTOF instrument. Thus, it might be said that spectra at low energy are typically dominated by "precursor" ions, while high energy spectra are dominated by "product" ions. Using a

single-TOF instrument, it is possible to modify source voltages to promote in-source fragmentation, and similar information might be acquired. However, the fragmentation data obtained are of reduced signal intensity, due to the formation of adducts and the effect of neutrals in the source [29]. In our own experience, we have also observed that mass accuracy deteriorates and less abundant fragmentation occurs, in comparison to the use of a collision cell. Hence, the MS^E approach (which produces collision cell fragmentation) is preferable for qualitative purposes. All MS data obtained by this approach are processed after acquisition, allowing the search for many contaminants, without additional analysis, simply by reprocessing the sample data. Despite these advantages, this technique is not true MS/MS (where only single precursor ions are fragmented). With MS^E, co-eluting components can enter the collision cell simultaneously, complicating interpretation (precursor–product ion relationships) from LE and HE mass spectra. For this reason, efficient chromatographic separation is important. This can be accomplished using UHPLC, which is also compatible with the high acquisition speed of TOF analyzers, minimizing matrix interferences and rendering high purity mass spectra, owing to high resolution separations.

The aim of this work is to explore the potential of combining UHPLC with accurate mass measurements of TOF-MS for the rapid screening of 76 illicit drugs, prescription drugs with potential for abuse, and their metabolites in wastewater samples. Using a QTOF instrument allows the collection of both protonated molecule and fragment ion information at accurate mass from a single analysis, as well as reprocessing of the samples to search for other interesting compounds, without additional analysis. Using the MS^E approach, we illustrate the detection and confident identification of several relevant compounds in difficult matrices like urban wastewater (influent and effluent).

2. Experimental

2.1. Reagents and chemicals

Illicit drugs and metabolites (amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, norcocaine, ketamine, morphine, codeine,

norcodeine, and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH, a metabolite of cannabis) were obtained from Sigma–Aldrich (St. Louis, MO, USA), Cerilliant (Round Rock, TX, USA), and the National Measurement Institute (Pymble, Australia) as solutions in methanol or acetonitrile, or as salt. Standard stock solutions of each compound were prepared at 100 mg L^{-1} in methanol or acetonitrile. Intermediate solutions (10 mg L^{-1}) were prepared by diluting the stock solution ten times with methanol. All standard solutions were stored in amber glass bottles at −20 °C.

HPLC-grade water was obtained by purifying demineralised water in a Milli-Q plus system from Millipore (Bedford, MA, USA). HPLC-grade methanol (MeOH), sodium hydroxide >99% (NaOH), ammonia solution (25%), and formic acid (98–100%) were acquired from Scharlau (Barcelona, Spain). Leucine enkephalin, used as the lock mass, and imazalil, used for calibration, were purchased from Sigma–Aldrich and Dr. Ehrenstorfer (Augsburg, Germany), respectively.

2.2. Instrumentation

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration–TOF mass spectrometer (Q-oaTOF Premier, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI interface operating in positive ion mode. The UPLC separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size analytical column 150 mm × 2.1 mm (Waters) at a flow rate of 300 µL min^{-1}. The mobile phases used were A = H_2O with 0.01% HCOOH and B = MeOH with 0.01% HCOOH. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10%; 14 min, 90%; 16 min, 90%; 16.01 min, 10%; 18 min, 10%. Nitrogen (from a nitrogen generator) was used as the drying gas and nebulizing gas. The gas flow was set at 600 L h^{-1}. TOF-MS resolution was approximately 10.000 at full width half maximum (FWHM) in V-mode and 17.500 FWHM in W mode, at *m/z* 556.2771. MS data were acquired over an *m/z* range of 50–1000. The microchannel plate (MCP) detector potential was set to 1850 V. A capillary voltage of 3.5 kV and cone voltage of 25 V were used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 350 °C and the source temperature to 120 °C. The column temperature was set to 60 °C.

For MS^E experiments, two acquisition functions with different collision energies were created. The first one, the low energy function (LE), selecting a collision energy of 4 eV, and the second one, the high energy (HE) function, with a collision energy ramp ranging from 15 eV to 40 eV in order to obtain a greater range of fragment ions. The LE and HE functions settings were for both a scan time of 0.2 s and an inter-scan delay of 0.05 s. For MS/MS experiments, a collision energy ramp from 15 to 40 eV was used, except for codeine (ramp from 25 to 65 eV). The automated attenuated function was also selected to correct for possible peak saturations (extended mode).

Calibrations were conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.05 M NaOH:5% HCOOH diluted (1:25) with acetonitrile:water (80:20), at a flow rate of 10 μL min^{-1}. For automated accurate mass measurement, the lock-spray probe was used, using as lockmass a solution of leucine enkephalin (2 μg mL^{-1}) in acetonitrile:water (50:50) at 0.1% HCOOH pumped at 30 μL min^{-1} through the lock-spray needle. A cone voltage of 65 V was selected to obtain adequate signal intensity for this compound (~500 counts). The protonated molecule of leucine enkephalin at *m/z* 556.2771 was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time. It should be noted that all the exact masses shown in this work have a deviation of 0.55 mDa from the "true" value, as the calculation performed by the MassLynx software uses the mass of hydrogen instead of a proton when calculating $[M+H]^+$ exact mass. However, because this deviation is also applied during mass axis calibration, there is no negative impact on the mass errors presented in this article. MS data were acquired in centroid mode and were processed by the ChromaLynx XS application manager (within MassLynx v 4.1; Waters Corporation).

2.3. Water samples

Twenty-four-hour composite influent and effluent water samples from three wastewater treatment plants (WWTP) located in the province of Castellon (Eastern Spain) were collected in polystyrene bottles (1 L) during the third week of June 2008 and the third week of July 2008. Samples were immediately stored at −20 °C until analysis. Before analysis, samples were thawed at room temperature.

2.4. Analytical procedure

A solid-phase extraction (SPE) step was applied based on our previous work [16]. Briefly, after shaking the water samples vigorously, they were centrifuged at 4500 rpm for 5 min to remove possible solid material. An aliquot of 50 mL of effluent wastewater or 50 mL of five-fold diluted influent wastewater sample was taken and its pH adjusted to 2.0 with formic acid. SPE was performed using Oasis MCX cartridges, previously conditioned by washing and rinsing with 6 mL of MeOH, 3 mL of Milli-Q water, and 3 mL of acidified water (pH 2). Samples were loaded onto the cartridges by gravity, and then cartridges were washed with 5 mL of 2% ammonia in water and vacuum dried for 5 min. Analytes were eluted using 8 mL of a 2% ammonia solution in MeOH. The eluate was evaporated to dryness at 35 °C under a gentle stream of nitrogen and reconstituted in 1 mL of 10:90 methanol:water. Analyses were performed by injecting 25 µL of the final extract into the UHPLC–QTOF MS system.

3. Results and discussion

3.1. UHPLC-QTOF MS screening method

As stated earlier, a large number of drugs may, in principle, be detected and identified. Obviously, compounds subjected to investigation will have to satisfy the requirements for LC–MS analysis. They should be satisfactorily ionized in the atmospheric pressure ionization (API) source employed (typically ESI), and they should not be lost along the sample treatment applied.

In this work, we have used 11 illicit drugs as model compounds, for which reference standards were available in our laboratory. This allowed us to establish the chromatographic and MS conditions most appropriate for their determination. These 11 compounds, with different physical properties, were previously studied by our research group, and a LC–MS/MS method using triple quadrupole-MS was developed for their quantification at sub-ppb levels in water [16].

The chromatographic system used in the present work employed an analytical UHPLC column of 150 mm in order to have improved separation and spectrum purity. Satisfactory results were obtained using 0.01% formic acid in both the aqueous and

organic components of the mobile phase, favouring the formation of the protonated molecule, and reducing the formation of adducts (sodium, potassium, ammonium) in positive ion-ESI. Since the majority of the compounds included in the screening possess a basic group, more abundant ionization in positive-ion mode would be expected. Consequently, negative ionization experiments were not carried out for simplification, although this acquisition mode might have permitted a greater number of candidates to be searched.

For a wide-scope screening, simplification of the sample treatment is desirable in order to include as many compounds as possible. From this point of view, direct injection would be ideal, as it would imply fully representative samples, avoiding potential losses of analytes that might occur with sample manipulation (e.g., SPE breakthrough, volatilization, etc.) New generations of QTOF instruments, with improved resolution and sensitivity, facilitate direct injection of aqueous samples for detection of illicit drugs. Nevertheless, sample treatment (mainly pre-concentration and/or clean-up) is still necessary for the determination of these compounds in water samples, because the low analyte concentrations and the complexity of the matrix, mainly in wastewater, which often result in matrix interferences. Illicit drugs have been generally isolated from aqueous samples by mixed reversed-phase cation-exchange SPE cartridges, using Oasis MCX or Strata XC™ sorbent [6, 7]. In our previous work [16], satisfactory results were obtained for the 11 pre-selected drugs, using Oasis MCX cartridges. Therefore, these cartridges have been also used in the present work, as it is reasonable to expect that, using the same sample pre-treatment, some other illicit drugs can be detected and identified in influent and effluent wastewater samples by QTOF-MS, running in MS^E acquisition mode.

3.2. Design of a compound database

LC–QTOF in MS^E acquisition mode is perfectly suited for wide-scope screening of illicit drugs in wastewater, as these compounds and their metabolites are mostly LC-amenable, and QTOF offers information useful for the reliable identification of compounds detected. In addition, data obtained from MS^E experiments could also be used to detect and identify other compounds of interest that were not included in the

initial list, without having available reference standards. By means of specialized software and a compound database, accurate mass information can be processed using mass error and isotopic fit. Since detection and identification is performed by using full MS acquisition data, data can be reprocessed and re-evaluated using new or modified databases to search for other interesting compounds, simply by including their theoretical mass and empirical formulae into the database.

The compound database used in this work is shown in **Table 1**. It included a total of 76 theoretical exact masses of (mostly) illicit drugs, prescription drugs with potential for abuse, and their metabolites. Analytes were selected mainly based on existing compound lists encountered in the literature, including compounds like nicotine and its metabolite cotinine. Although not being considered as illicit drug, millions of people have developed nicotine dependency. So, it was considered as a possible emerging contaminant, and therefore, also added to our database. As 11 reference standards were available in our laboratory, their experimental data (retention time and in-source fragment ions) were used for rapid detection and more confident identification. This allows the search for target compounds also using the fragment ions in those particular cases where abundant in-source fragmentation occurs. For the remaining 65 compounds, only the theoretical mass and empirical formulae was included in the database.

ChromaLynx XS (software provided by Waters) offers the possibility of applying a "post-target" processing method based on selected exact masses that permits a rapid and simple reviewing by cataloguing analytes, as a function of mass error. In addition, this software allows the simultaneous visualization of the complete spectrum of positive findings.

Table 1. Compound database of illicit drugs, prescription drugs with potential for abuse and metabolites.

Compound	Empirical formulae	Exact mass $[M+H]^+$	Compound	Empirical formulae	Exact mass $[M+H]^+$
10-hydroxymorphine	$C_{17}H_{19}NO_4$	302.1392	Ketamine	$C_{13}H_{16}NOCl$	238.0999
11-hydroxy-THC (OH-THC)	$C_{21}H_{30}O_3$	331.4778	Lysergic acid diethylamide (LSD)	$C_{20}H_{25}N_3O$	324.2076
11-hydroxy-THC glucuronide	$C_{27}H_{38}O_9$	507.2594	Mescaline	$C_{11}H_{17}NO_3$	212.1286
11-nor-9-carboxy-Δ9-THC (THC-COOH)	$C_{21}H_{28}O_4$	345.2066	Methadone	$C_{21}H_{27}NO$	310.2178
11-nor-9-carboxy-Δ9-THC glucuronide	$C_{27}H_{36}O_{10}$	521.2387	Methamphetamine (MA)	$C_{10}H_{15}N$	150.1282
14-hydroxycodeine	$C_{18}H_{21}NO_5$	332.1498	Methcathinone	$C_{10}H_{13}NO$	164.1075
2-ethylidene-1,5-dimethyl-3,3-diphenyl-pyrrolidine (EDDP)	$C_{20}H_{23}N$	278.1909	Methylecgonine	$C_{10}H_{17}NO_3$	200.1286
2-oxo-3-hydroxy-LSD	$C_{20}H_{25}N_3O_3$	356.4478	Methylecgonidine	$C_{10}H_{15}NO_2$	182.1181
3,4-methylenedioxy amphetamine (MDA)	$C_{10}H_{13}NO_2$	180.1024	m-Hydroxy benzoylecgonine	$C_{16}H_{19}NO_5$	306.1341
3,4-methylenedioxyethyl amphetamine (MDEA)	$C_{12}H_{17}NO_2$	208.1337	Morphine	$C_{17}H_{19}NO_3$	286.1443
3,4-methylenedioxymeth amphetamine (MDMA)	$C_{11}H_{15}NO_2$	194.1181	Morphine-3β-D glucuronide (M3G)	$C_{23}H_{27}NO_9$	462.1764
3-acetylmorphine (3-AM)	$C_{19}H_{21}NO_4$	328.1549	Morphine-6β-D glucuronide (M6G)	$C_{23}H_{27}NO_9$	462.1764
3'-hydroxycocaine	$C_{17}H_{21}NO_5$	320.1498	Morphine-7,8-oxide	$C_{17}H_{19}NO_4$	302.1392
6-acetylmorphine (6-AM)	$C_{19}H_{21}NO_4$	328.1549	Morphine-GSH	$C_{27}H_{34}N_4O_9S$	591.2125
7-aminoflunitrazepam	$C_{16}H_{14}FN_3O$	284.1199	N-Hydroxynorcocaine	$C_{16}H_{19}NO_5$	306.1341
Acetylcodeine	$C_{20}H_{23}NO_4$	342.1705	Nicotine	$C_{10}H_{14}N_2$	163.1235
Amphetamine (AM)	$C_9H_{13}N$	136.1126	norBenzoylecgonine	$C_{15}H_{17}NO_4$	276.1236
Benzoylecgonine	$C_{16}H_{19}NO_4$	290.1392	norCocaethylene	$C_{17}H_{21}NO_4$	304.1549
Cocaethylene	$C_{18}H_{23}NO_4$	318.1705	norCocaine	$C_{16}H_{19}NO_4$	290.1392
Cocaine	$C_{17}H_{21}NO_4$	304.1549	norCodeine	$C_{17}H_{19}NO_3$	286.1443
Codeine	$C_{18}H_{21}NO_3$	300.1599	norLSD	$C_{19}H_{23}N_3O$	310.1919
Codeine epoxide	$C_{18}H_{21}NO_4$	316.1549	norMorphine	$C_{16}H_{17}NO_3$	272.1286
Codeine-6 glucuronide	$C_{24}H_{29}NO_9$	476.1921	norMorphine-6 glucuronide	$C_{22}H_{25}NO_9$	448.1608
Cotinine	$C_{10}H_{12}N_2O$	177.1028	norTropacocaine	$C_{14}H_{17}NO_2$	232.1337
Desmethyldiazepam	$C_{15}H_{11}ClN_2O$	271.0638	Oxazepam	$C_{15}H_{11}ClN_2O_2$	287.0587
Diazepam	$C_{16}H_{13}ClN_2O$	285.0794	Oxycodone	$C_{18}H_{21}NO_4$	316.1549
Dihydrocodeine	$C_{18}H_{23}NO_3$	302.1756	Oxymorphone	$C_{17}H_{19}NO_4$	302.1392
Dihydromorphine	$C_{17}H_{21}NO_3$	288.1599	Paramorphine	$C_{19}H_{21}NO_3$	312.1599
Ecgonidine	$C_9H_{13}NO_2$	168.1025	Pentobarbital	$C_{11}H_{18}N_2O_3$	227.1395
Ecgonine	$C_9H_{15}NO_3$	186.1130	Phenacetine	$C_{10}H_{13}NO_2$	180.1024
Ephedrine	$C_{10}H_{15}NO$	166.1232	Phencyclidine (PCP)	$C_{17}H_{25}N$	244.2065
Ethylecgonine	$C_{11}H_{19}NO_3$	214.1443	Phenobarbital	$C_{12}H_{12}N_2O_3$	233.0926
Fentanyl	$C_{22}H_{28}N_2O$	337.2280	Pseudomorphine	$C_{34}H_{36}N_2O_6$	569.2651
Flunitrazepam	$C_{16}H_{12}FN_3O_3$	314.0941	Pseudomorphine (C17 alkaloid)	$C_{17}H_{19}NO_3$	286.1443
γ-hydroxybutyric acid (GHB)	$C_4H_8O_3$	105.0551	Psicolin	$C_{12}H_{16}N_2O$	205.1341
Heroin	$C_{21}H_{23}NO_5$	370.1654	Psilocybin	$C_{12}H_{17}N_2O_4P$	285.1004
Hydrocodone	$C_{18}H_{21}NO_3$	300.1599	Temazepam	$C_{16}H_{13}ClN_2O_2$	301.0744
Hydromorphone	$C_{17}H_{19}NO_3$	286.1443	Tetrahydrocannabinol (THC)	$C_{21}H_{30}O_2$	315.2324

3.3. Elucidating isomeric compounds

Orthogonal acceleration TOF-MS instruments permit measurements with mass accuracy commonly better than 5 ppm and mass resolution exceeding 10.000 FWHM. The mass of any ionizable component in different sample matrices can be measured accurately, which gives high confidence to the identification process, and also allows the possibility of distinguishing between isobaric compounds. Isobars have identical nominal mass, but different elemental composition, and thus, different exact mass. By discriminating isobaric compounds from differences in mass defect, TOF-MS allows reducing the possibilities of false identification.

In contrast, isomeric compounds have identical elemental composition, and thus, exact mass. Retention time measurements and acquisition of fragment ions are two common tools that allow isomer differentiation. **Fig. 1** shows MS/MS chromatograms for two isomers, morphine and norcodeine ($[M+H]^+$, *m*/z 286.1443), together with their MS^E and MS/MS spectra. As reference standards were available to the authors and chromatographic separation was satisfactory, discrimination of both analytes could be made from their retention times (morphine 1.61 min, norcodeine 2.49 min). However, reference standards would have not been indispensable using QTOF MS, as these two isomers could also be discriminated by specific fragment ions. Raith et al. [33] gave a detailed description of the complex fragmentation pattern of morphine and related compounds. It can be seen in **Fig. 1** that morphine and norcodeine share most of their abundant ions in their MS/MS or HE TOF-MS spectra. However, a few specific fragments can be found in their spectra. For example, morphine shows a *m*/z 229 ion, which would correspond to the loss of $CH_2 = CHNHCH_3$; further loss of H_2O or CO would lead to fragment ions *m*/z 211 and 201, respectively. For norcodeine, *m*/z 225 would correspond to the loss of $CH_2 = CHNH_2$ and H_2O. In addition, minor fragments at *m*/z 243 (loss of $CH_2 = CHNH_2$) and *m*/z 215 (loss of $CH_2 = CHNH_2$ and CO) are also present, but considerably less abundant.

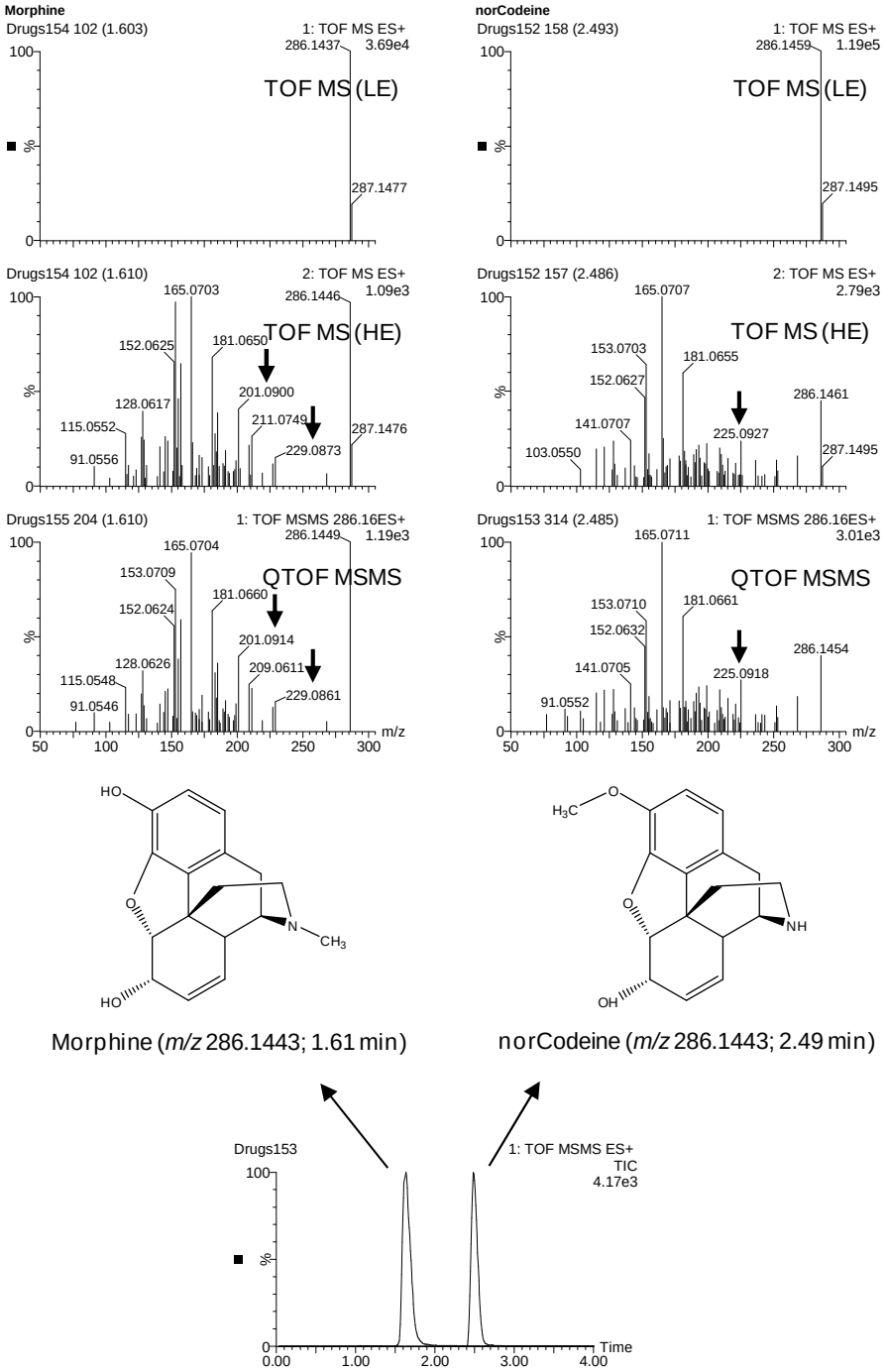

Figure 1. Fragmentation and identification of the isomeric compounds morphine and norcodeine by UHPLC-QTOF-MS. Specific fragments are indicated with an arrow in the spectra.

In the same way, another couple of isomers, benzoylecgonine and norcocaine ([M+H]$^+$, *m*/z 290.1392) could be discriminated by their specific fragment ions. For benzoylecgonine these were *m*/z 150 and 82, whereas norcocaine showed characteristic fragment ions at *m*/z 136 and 68 (data not shown).

From these and other examples, we observed that HE MS^E and MS/MS have similar capabilities, as the ions present in their spectra and the relative abundances are highly comparable. This implies that by performing QTOF-MS experiments in the HE MS^E acquisition mode, both protonated molecule and fragmentation data could be obtained from a single injection, without the need of preselecting a precursor ion, a notable difference compared with "true" QTOF-MS/MS experiments.

3.4. MS^E screening of environmental samples

Twenty urban wastewater samples (10 influents and 10 effluents) were analyzed by UHPLC–QTOF MS. The identification criteria included a narrow mass window (nw) of 0.01 Da, (±0.005 Da), an area of at least 50 a.u. (arbitrary units), and a retention time window of ±0.2 min, the latter being applied only when the reference standard was available.

After QTOF-MS screening, several positive findings could easily be identified and confirmed with reference standards (and therefore, accurate mass spectra and retention times). For example, **Fig. 2 top** shows the narrow window-XIC chromatograms, and LE and HE TOF-MS spectra for three effluent wastewater samples, where benzoylecgonine, MDMA, and methamphetamine were detected. It illustrates that a highly reliable identification of the compound detected could be made using the MS^E approach. This figure also shows MS spectra for their reference standards (**Fig. 2 bottom**). The HE spectra of benzoylecgonine show the specific fragments *m*/z 150 and 82, as earlier noticed. This fact is relevant for the correct identification of this compound. Regarding MDMA, this compound could be identified since the "precursor" ion at accurate mass ([M+H]$^+$, *m*/z 194.1198) was present in the LE spectra. Thus, its HE spectra could not be confused with MDA or MDEA, which have similar fragmentation patterns, but different "precursor" ions [6, 16]. At high energy, the protonated molecule

of methamphetamine ($[M+H]^+$, *m*/z 150.1283) was almost completely fragmented, mainly to the *m*/z 91 ion. Due to the low analyte level in the sample, it was difficult to identify methamphetamine based on HE spectra. However, the LE spectra showed the protonated molecule and two characteristic fragments with mass errors below 0.5 mDa, giving high confidence to the identification of this compound.

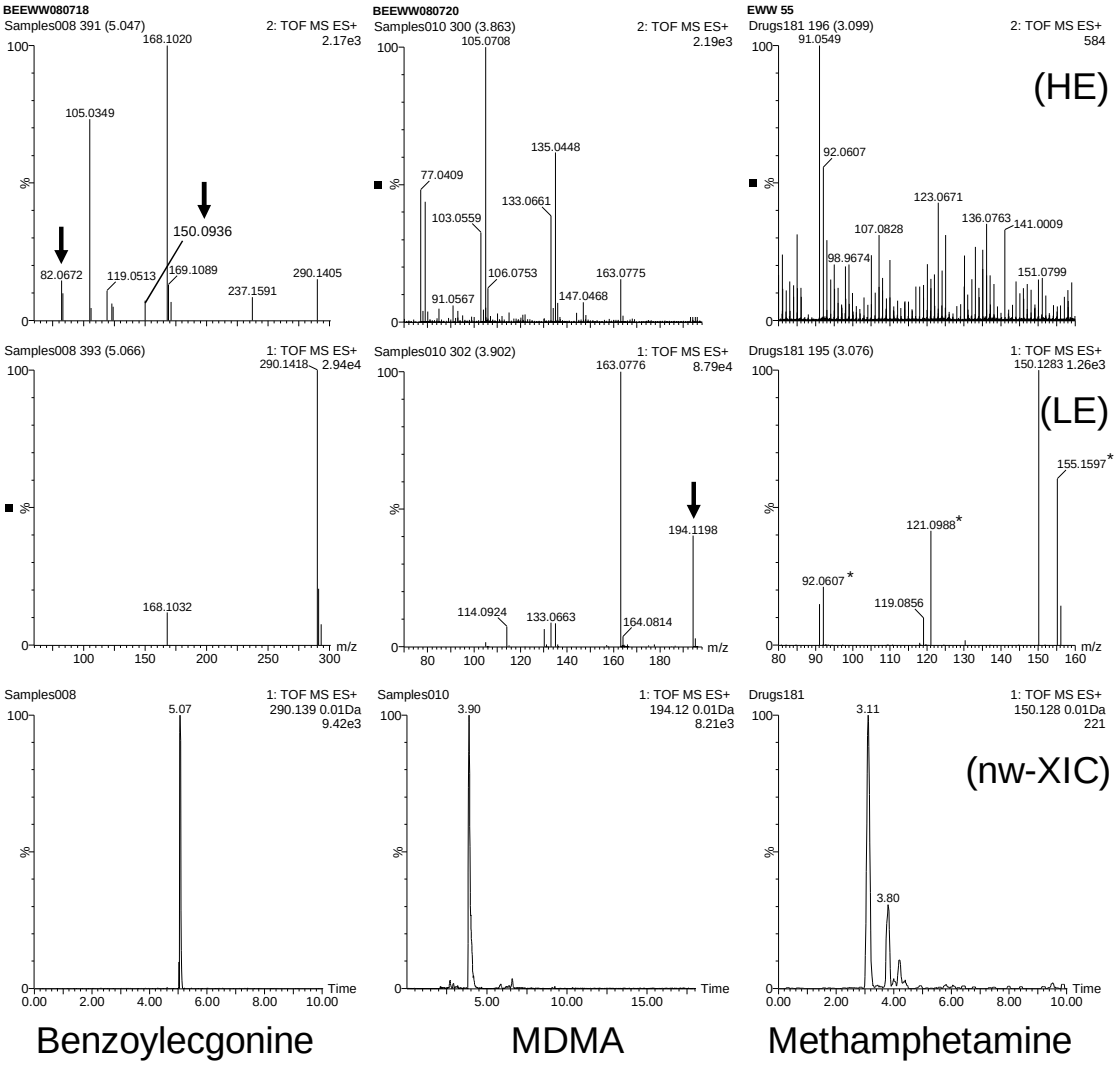

Figure 2. Identification by UHPLC-QTOF operating in MS^E acquisition mode of benzoylecgonine, MDMA, and methamphetamine. ***Effluent wastewater (top)*** reference standards (bottom).

* Fragments of the deuterated analogue, which was added to this sample.

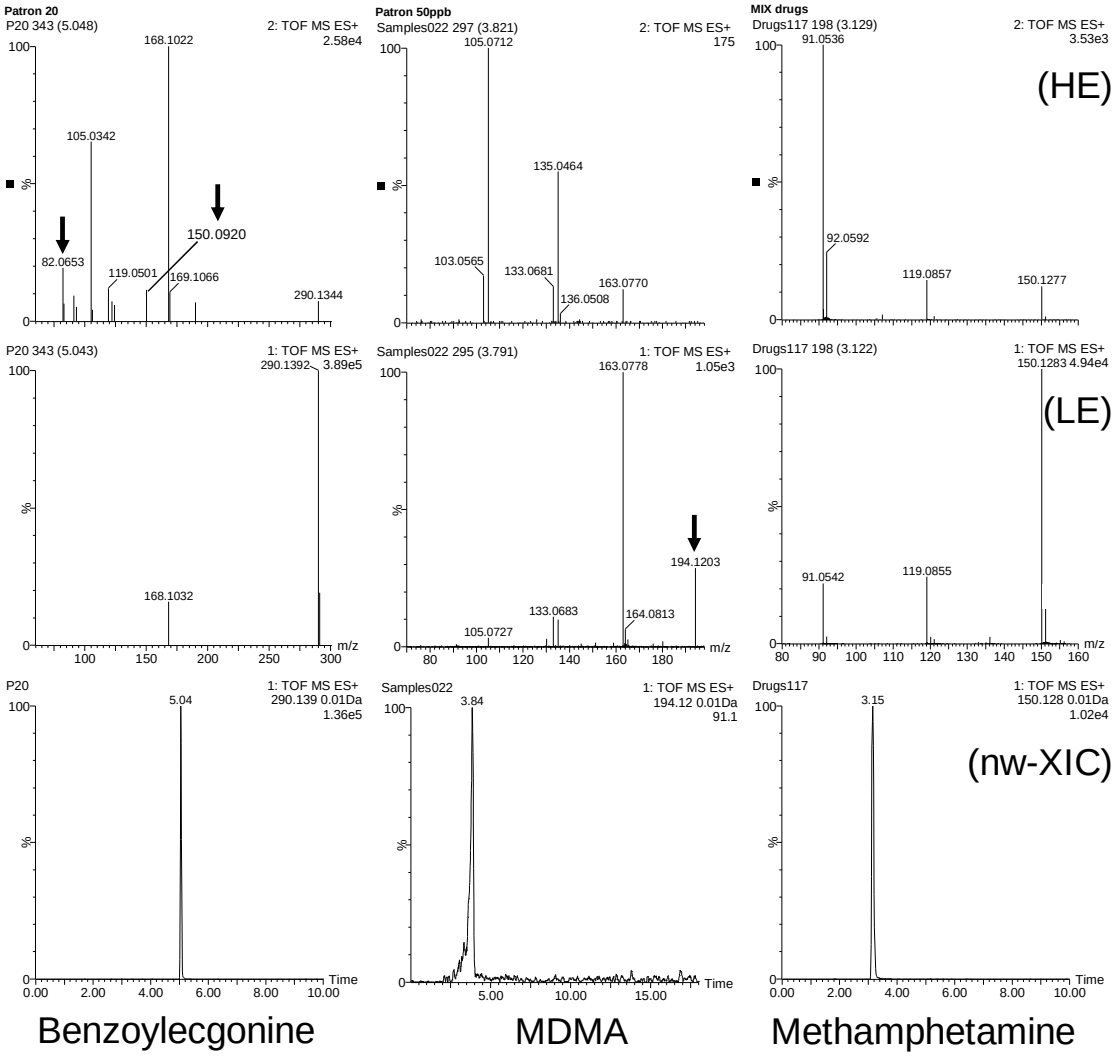

Figure 2. Identification by UHPLC-QTOF operating in MS^E acquisition mode of benzoylecgonine, MDMA, and methamphetamine. Effluent wastewater (top) ***reference standards (bottom)***.

As stated earlier, specific software (ChromaLynx XS, Waters) permits the rapid and simple review of selected compounds in a "post-target" way, based on the accurate mass of (typically) the protonated molecule $[M+H]^+$. It allows visualization of the nw-XIC chromatogram and LE TOF-MS spectrum of positive findings. Moreover, the retention time, mass error, and i-FIT of each potential positive are shown. For example, oxazepam was detected in an effluent wastewater sample (**Fig. 3**). Oxazepam is a

benzodiazepine, a prescriptional drug with potential for abuse or dependency. Additional information could be obtained by manual extraction of the corresponding HE spectra. Accurate masses of fragments and associated neutral losses of the suspected oxazepam could be justified from its chemical structure. The isotope fit, based on the isotopic distribution and accurate masses, is also valuable parameter when identifying a compound. In the case of oxazepam, an excellent i-Fit value (0.00) was obtained, taking into account the distribution of the ^{37}Cl isotope. This characteristic isotopic pattern can also be observed for the fragments *m/z* 269, 241, and 163. They could, therefore, easily be linked to oxazepam, since there was no loss of chlorine and they maintained the same isotopic distribution. In a similar way, ketamine was also detected and identified in urban wastewater with high confidence (data not shown).

Another interesting example is the detection of codeine in influent and effluent samples. Promoting fragmentation by MS^E and using the default HE collision ramp of 15–40 eV, the protonated molecule ($[M+H]^+$, *m/z* 300.1618) was still present in the HE TOF-MS spectrum at high abundance, together with a small fragment ion (*m/z* 165.0731) (**Fig. 4a**). Although mass errors were low (1.9 and 2.7 mDa, respectively) and the *m/z* 165 fragment ion could be justified, a reference standard was purchased to confirm its identity. Using the codeine standard, additional QTOF-MS/MS experiments could be carried out. QTOF spectra in tandem MS mode are "cleaner" and fragmentation is more efficient, due to isolation of the precursor ion and the optimization of both cone and collision energy. In contrary to methamphetamine, where the "precursor" ion was almost completely fragmented at a default HE of 15–40 eV (see **Fig. 2**), the protonated molecule of codeine hardly fragmented under these conditions. Therefore, QTOF-MS/MS experiments were carried out with an increased collision energy ramp of 25–65 eV for both the positive sample and the standard (**Fig. 4b**). Fragmentation of the precursor ion was more efficient, leading to useful information for confirmation. Similar QTOF-MS/MS spectra are shown for both the sample and the standard. As expected, fragmentation was similar to other opioids (see **Fig. 1**), in agreement with the fragmentation pathway described by Raith et al. [33]. In addition, the retention times for the sample and standard did match, which again confirms that the compound detected was codeine.

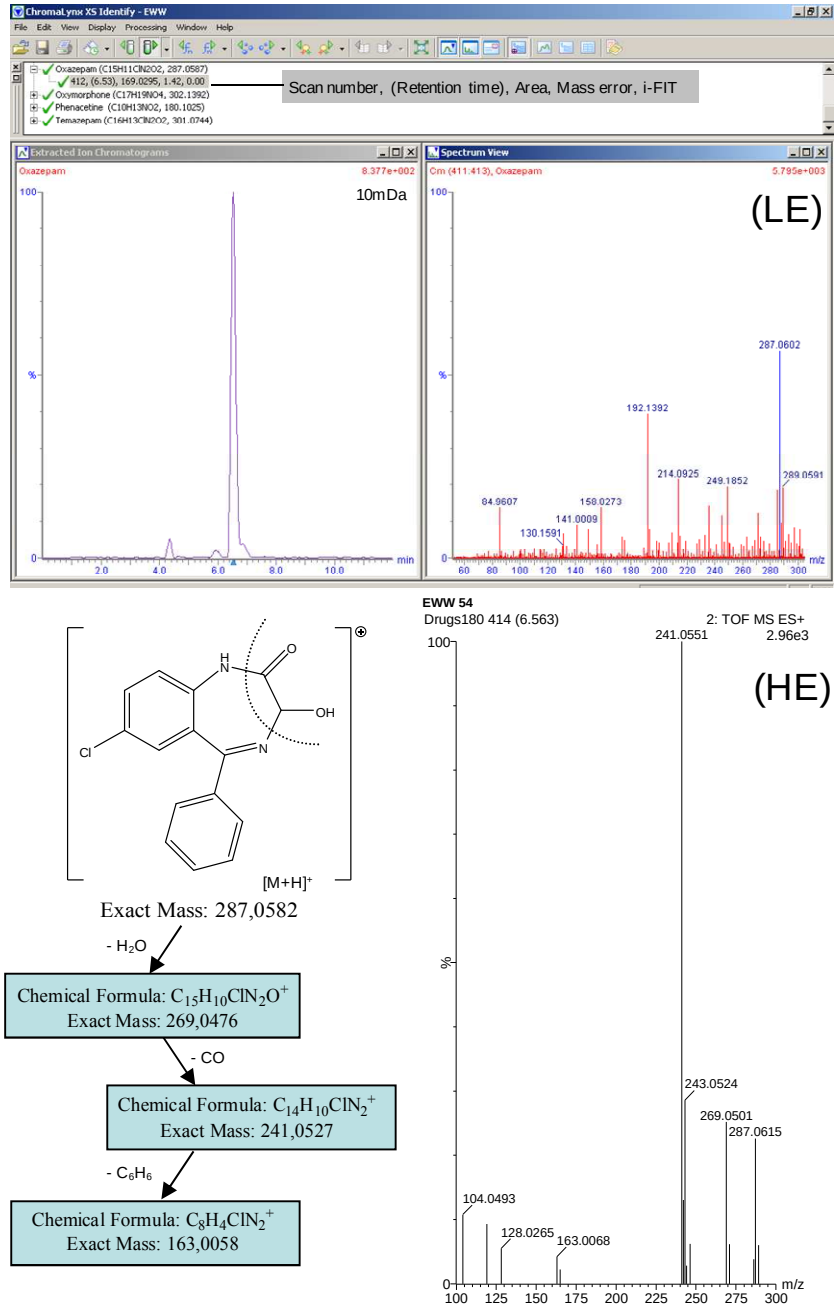

Figure 3. Detection and identification of oxazepam in effluent wastewater. Chromatrogram and LE spectrum of the sample as presented by ChromaLynx XS (top). HE spectrum (bottom right). Structure of oxazepam and justification of its most abundant fragments (bottom left).

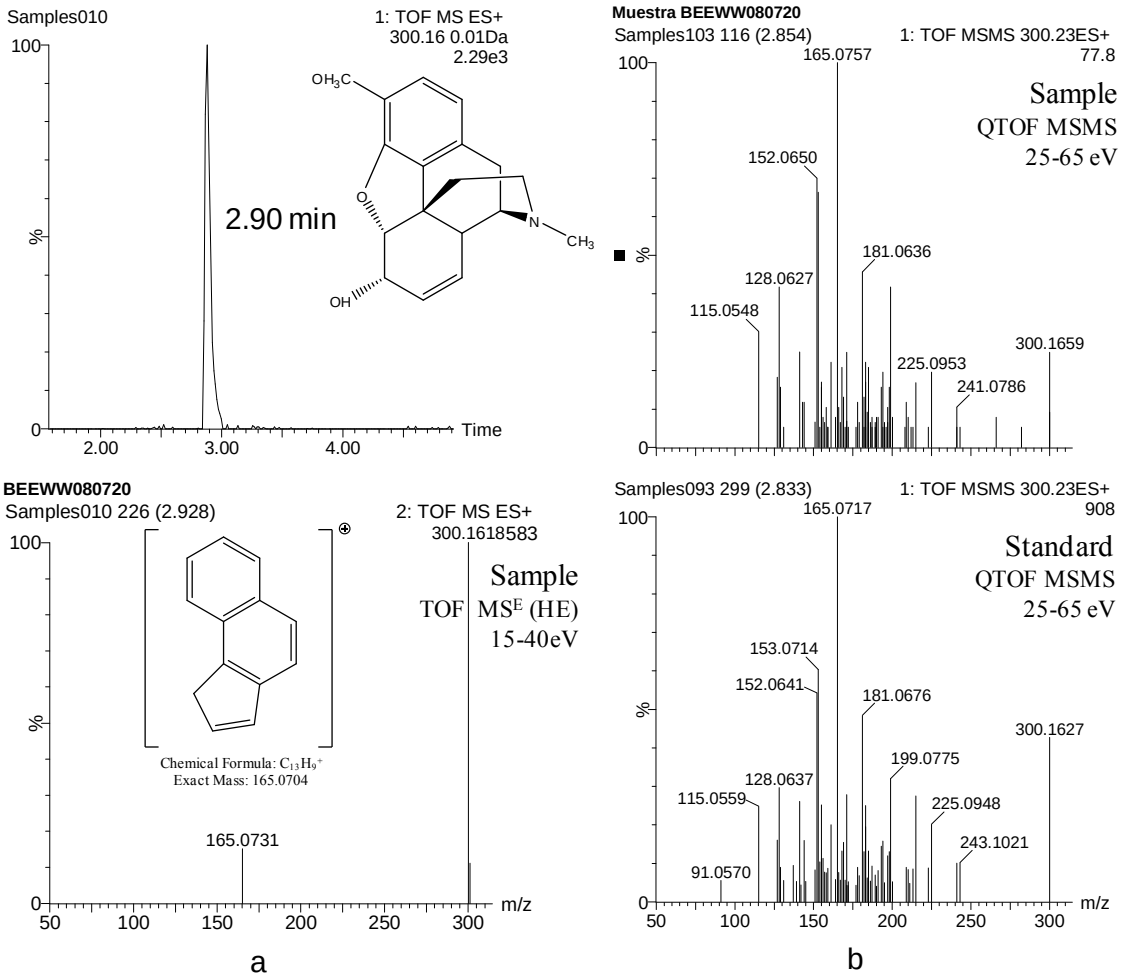

Figure 4. Detection and identification of codeine in effluent wastewater by UHPLC-QTOF-MS. (a) Chromatrogram and HE spectrum of the sample applying a default collision energy ramp 15 – 40 eV (b) MS/MS spectra (collision energy ramp of 25 – 65 eV) for the sample (top) and reference standard (bottom).

Using the proposed methodology, a confident identification of the compounds detected in samples can be made on the basis of QTOF accurate MS data, even without reference standards. This is surely acceptable for chemical screening. However, when legal or societal implication is involved, the unequivocal identification provided by comparison to an analytical standard should be done. In any case, the acquisition of additional reference standards would be required only when solid evidence

provided by QTOF MS exists on the presence of a given compound. This would allow avoiding the high cost and the inconvenience of buying all 76 standards.

A summary of the drugs detected in the 20 wastewater samples analyzed (ten influent and ten effluent samples) is shown in **Table 2**. Ten compounds were detected and identified following the methodology described in this article. Compounds most frequently found in influent wastewater were cocaine and its major metabolite benzoylecgonine, together with codeine and the nicotine metabolite cotinine. Codeine, benzoylecgonine, and oxazepam were the most widely drugs detected in effluent wastewater.

Table 2. Drugs detected in influent (IWW) and effluent wastewater (EWW) samples.

Compounds	Number of detections IWW (n=10)	Average absolute mass error (mDa)	Number of detections EWW (n=10)	Average absolute mass error (mDa)
Benzoylecgonine	10	0.9	6	1.9
Cocaine	8	1.9	2	1.2
Codeine	10	2.5	8	1.5
Cotinine	10	1.7	-	-
Ketamine	-	-	2	1.9
MDMA	3	0.7	3	1.3
Methamphetamine	-	-	1	0.1
norBenzoylecgonine	-	-	1	4.6
Oxazepam	2	1.7	8	1.3
Temazepam	-	-	3	1.7

Although the objective of this paper was to explore UHPLC–QTOF MS for rapid qualitative screening of drugs of abuse, an estimation of the detectable concentration levels could be made from the instrumental detection limits (IDLs) only in those cases where reference standards were available (11 compounds). In addition, comparative QqQ data of wastewater samples were available for 5 out of 11 model compounds, since quantitative UHPLC–MS/MS analyses [16] were also performed after screening. These five compounds (MDMA, methamphetamine, cocaine, benzoylecgonine and norbenzoylecgonine) were found by both QTOF and QqQ methodologies in influent and/or effluent wastewater. Regarding IDLs, they were statistically estimated, for a signal-to-noise of 3, from individual reference standards in solvent. IDLs estimated were around 1.0 ppb for amphetamine and amphetamine-type stimulants, 0.3 ppb for cocaine and metabolites (except for benzoylecgonine (0.01 ppb) and

norbenzoylecgonine (0.1 ppb)), and 3.5 ppb for THC-COOH. Taking into account the sample pre-concentration factor, and with all preventions owing to matrix effects, the IDLs values would roughly be 10 and 50 times lower in influent and effluent wastewater, respectively. Quantitative QqQ data allowed estimation of concentration levels in real-time wastewater samples. Thus, MDMA, cocaine and benzoylecgonine at concentrations (reported by QqQ) of 3; 0.6 and 2 ppb, respectively, in an influent wastewater sample could be detected and confirmed by QTOF MS operating in MS^E mode. Moreover, MDMA, methamphetamine, cocaine, benzoylecgonine and norbenzoylecgonine at 0.5 ppb (reported by QqQ) could also be detected and confirmed by QTOF MS in effluent wastewater.

4. Conclusions

This paper demonstrates that UHPLC–QTOF MS is a powerful technique for screening purposes. Its application to monitoring drugs of abuse in urban wastewater has allowed the detection and identification of several widely consumed illicit drugs, together with other drugs that could be potentially used for illicit consumption. The use of a QTOF instrument has allowed the fragmentation of the analyte molecule in the collision cell, working in TOF-MS mode. Moreover, operating the instrument in MS^E acquisition mode it is feasible to simultaneously obtain full-spectrum MS data at accurate mass at low and high collision energy. The combination of these two datasets is highly useful for identification and elucidation purposes, as LE MS spectra usually show the protonated molecule (in positive-ESI), while HE MS spectra are rather rich in abundance of fragment ions. Taking into account all information provided by this technique (accurate mass, isotopic distribution, and MS data at LE and HE), it is feasible to identify compounds in complex environmental samples, even without using reference standards, by searching for target analytes on the basis of a compound database. Development of MS^E spectra libraries would be highly useful for future works to facilitate this task.

In this work, the screening was carried out for 76 compounds, the majority being drugs of abuse and their metabolites. However, since screening and identification is performed after full-MS acquisition, data can be reprocessed to search for other interesting compounds at any time, without the need for additional analysis of the sample. Although not applied in this work, this technique allows for the possibility to identify unknown compounds, like non-selected metabolites or transformation products (TPs) or, in general, non-target contaminants [34] using the same accurate mass data and relevant information given by QTOF-MS analysis. This demonstrates the potential of this technique for screening organic pollutants in environmental waters.

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References

[1] S.D. Richardson, Water analysis: emerging contaminants and current issues, Anal. Chem. 81 (2009) 4645 - 4677.

[2] S.D. Richardson, Environmental mass spectrometry: emerging contaminants and current issues, Anal. Chem. 82 (2010) 4742 - 4774.

[3] T.L. Jones-Lepp, D.A. Alvarez, J.D. Petty, J.N. Huckins, Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents, Arch. Environ. Contam. Toxicol. 47 (2004) 427-439.

[4] E. Zuccato, C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea, R. Fanelli, Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse, Environ. Health 4:14 (2005) http://www.ehjournal.net/content/4/1/14.

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

[6] S. Castiglioni, E. Zuccato, C. Chiabrando, R. Fanelli, R. Bagnati, Mass spectrometric analysis of illicit drugs in wastewater and surface water, Mass Spectrometry Reviews 27 (2008) 378 - 394.

[7] C. Postigo, M.J. López de Alda, D. Barceló, Analysis of drugs of abuse and their human metabolites in water by LC-MS^2: A non-intrusive tool for drug abuse estimation at the community level, Trends Anal. Chem. 27 (2008) 1053 - 1069.

[8] M. Huerta-Fontela, M.T. Galcerán, J. Martín-Alonso, F. Ventura, Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain, Sci. Tot. Env. 397 (2008) 31 - 40.

[9] E. Zuccato, S. Castiglioni, R. Bagnati, C. Chiabrando, P. Grassi, R. Fanelli, Illicit drugs a novel group of environmental contaminants, Water Res. 42 (2008) 961 - 968.

[10] C.A. Mueller, W. Weinmann, S. Dresen, A. Schreiber, M. Gergov, Development of a multi-target screening analysis for 301 drugs using a Qtrap liquid chromatography/tandem mass spectrometry system and automated library searching, Rapid Commun. Mass Spectrom. 19 (2005) 1332 – 1338.

[11] A.C. Hogenboom, J.A. van Leerdam, P. de Voogt, Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography - hybrid linear ion trap Orbitrap mass spectrometry, J. Chromatogr. A. 1216 (2009) 510 – 519.

[12] S. Kern, K. Fenner, H.P. Singer, R.P. Schwarzenbach, J. Hollender, Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry, Environ. Sci. Technol. 43 (2009) 7039-7046.

[13] M. Huerta-Fontela, M.T. Galcerán, F. Ventura, Ultraperformance liquid chromatography-tandem mass spectrometry analysis of stimulatory drugs of abuse in wastewater and surface waters, Anal. Chem. 79 (2007) 3821 – 3829.

[14] M. R. Boleda, M.T. Galcerán, F. Ventura, Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. A 1175 (2007) 38 - 48.

[15] B. Kasprzyk-Hordern, R.M. Dinsdale, A.J. Guwy, Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography-electrospray tandem mass spectrometry, Anal. Bioanal. Chem. 391 (2008) 1293 - 1308.

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

[17] A.C. Hogenboom, W.M.A. Niessen, D. Little, U.A.Th. Brinkman, Accurate mass determination for the confirmation and identification of organic microcontaminants in surface water using on-line solid-phase extraction liquid chromatography electrospray orthogonal-acceleration time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 13 (1999) 125 – 133.

[18] M. Petrovic, M. Gros, D. Barceló, Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry, J. Chromatogr. A. 1124 (2006) 68 - 81.

[19] J.V. Sancho, O.J. Pozo, M. Ibáñez, F. Hernández, Potential of liquid chromatography/time-of-flight mass spectrometry for the determination of pesticides and transformation products in water, Anal. Bioanal. Chem. 386 (2006) 987 – 997.

[20] M. Ibáñez, J.V. Sancho, D. McMillan, R. Rao, F. Hernández, Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-flight mass spectrometry, Trends Anal. Chem. 27 (2008) 481 - 489.

[21] M. Ibáñez, C. Guerrero, J.V. Sancho, F. Hernández, Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry, J. Chromatogr. A. 1216 (2009) 2529 - 2539.

[22] S. Ojanperä, A. Pelander, M. Pelzing, I. Krebs, E. Vuori, I. Ojanperä, Isotopic pattern and accurate mass determination in urine drug screening by liquid chromatography/time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 20 (2006) 1161 – 1167.

[23] A. Pelander, I. Ojanperä, S. Laks, I. Rasanen, E. Vuori, Toxicological screening with formula-based metabolite identification by liquid chromatography/time-of-flight mass spectrometry, Anal. Chem. 75 (2003) 5710 – 5718.

[24] F. Badoud, E. Grata, L. Perrenoud, L. Avois, M. Saugy, S. Rudaz, J.-L. Veuthey, Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography-

quadrupole time-of-flight mass spectrometry I. Screening analysis, J. Chromatogr. A 1216 (2009) 4423 - 4433.

[25] S. Grimalt, J.V. Sancho, O.J. Pozo, F. Hernández, Quantification, confirmation and screening capability of UHPLC coupled to triple quadrupole and hybrid quadrupole time-of-flight mass spectrometry in pesticide residue analysis, J. Mass Spectrom. 45 (2010) 421-436.

[26] M. Mezcua, O. Malato, J.F. García-Reyes, A. Molina-Díaz, A.R. Fernández-Alba, Accurate-mass databases for comprehensive screening of pesticide residues in food by fast liquid chromatography time-of-flight mass spectrometry, Anal. Chem. 81 (2009) 913 –929.

[27] I. Ferrer, A. Fernández-Alba, J.A. Zweigenbaum, E.M. Thurman, Exact-mass library for pesticides using a molecular-feature database, Rapid Commun. Mass Spectrom. 20 (2006) 3659 – 3668.

[28] J. Castro-Pérez, J. Hoyes, H. Major, S. Preece, Advances in MS-based approaches for drug and metabolism studies, Chromatographia supplement 55 (2002) 59 – 63.

[29] R.S. Plumb, K.A. Johnson, P. Rainville, B.W. Smith, I.D. Wilson, J. Castro-Pérez, J.K. Nicholson, UPLC/MS^E; a new approach for generating molecular fragment information for biomarker structure elucidation, Rapid Commun. Mass Spectrom. 20 (2006) 1989 – 1994.

[30] P.J. Weaver, A. Laures, J-C. Wolff, Investigation of the advanced functionalities of a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer, Rapid Commun. Mass Spectrom. 21 (2007) 2415 – 2421.

[31] P.R. Tiller, S. Yu, J. Castro-Pérez, K.L. Fillgrove, T.A. Baillie, High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line' approach for metabolite identification studies, Rapid Commun. Mass Spectrom. 22 (2008) 1053 – 1061.

[32] F. Hernández, S. Grimalt, O.J. Pozo, J.V. Sancho, Use of ultra-high-pressure liquid chromatography-quadrupole time-of-flight MS to discover the presence of pesticide metabolites in food samples, J. Sep. Sci. 32 (2009) 2245 - 2261.

[33] K. Raith, R. Neubert, C. Poeaknapo, C. Boettcher, M.H. Zenk, J. Schmidt, Electrospray tandem mass spectrometric investigations of morphinans, J. Am. Soc. Mass Spectrom. 14 (2003) 1262 – 1269.

[34] M. Ibáñez, J.V. Sancho, O.J. Pozo, W.M.A. Niessen, F. Hernández, Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water, Rapid Commun. Mass Spectrom. 19 (2005) 169 – 178.

Chapter 3.2.2, scientific article 4

Wide-scope screening of illicit drugs in urban wastewater by UHPLC-QTOF MS

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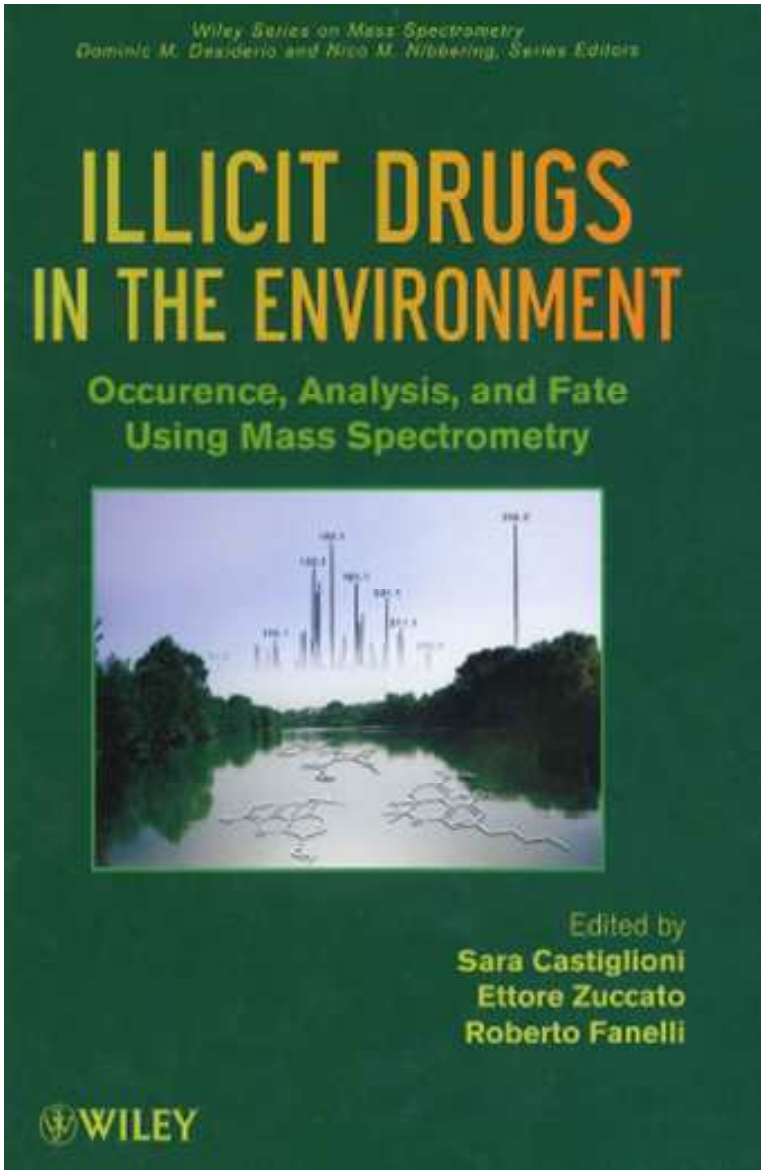
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4.1. TOF and QTOF MS analysis

The application of time-of-flight (TOF) mass spectrometry (MS) to environmental analyses has tremendously increased over the last decade. The sensitive full spectrum data acquisition and high-mass accuracy of new generation TOF instruments together with their high-mass resolving power provide a high degree confidence for identification of compounds detected in samples. The application of orthogonal acceleration (oa-)TOF MS instruments minimizes the possibility of reporting false positives because of the accurate mass measurement of (typically) the (de)protonated molecules. This is especially relevant when facing to highly complex environmental samples like urban wastewater. Confirmatory analysis, even with greater reliability, can be performed by using a hybrid quadrupole-TOF (QTOF) MS, where an analyte *m*/z ion can be preselected in the first quadrupole as precursor ion. When carrying out MS/MS experiments, the fragmentation of the precursor ion results in full-product ion spectra at accurate mass giving relevant structural information and extra confidence to confirm or deny a suggested structure.

In addition to their excellent potential in the qualitative field, TOF instruments offer the possibility of a wide-scope screening once the analysis has been performed and MS data acquired, because the preselection of analytes is not compulsory and searching for contaminants is made in a subsequent step. Ideally, screening methods should be able to rapidly detect the presence of as many contaminants as possible in a sample, preferably with little sample manipulation. As expected, in environmental MS, the instrumentation available is of particular importance when selecting the screening approach. Thus, TOF instruments allow performing target screening after MS acquisition, taking advantage of their sensitivity in full acquisition mode and the mass accuracy measurements. However, when analytes have to be preselected before acquisition (as in target methods based on LC-MS/MS with triple quadrupole), there is a need of reference standards of each target compound for MS optimization and many other pollutants (non-preselected) that might be present in samples would be ignored in the analysis.

For a wide-scope screening in environmental MS using TOF instruments, two alternatives can be typically applied (Hernández et al. 2005a; Ibáñez et al. 2008):

1. Post-target screening – after full-spectrum acquisition, selected *m/z* ions for the target compounds are extracted from the Total Ion Current (TIC) chromatogram, giving rice to eXtracted Ion Chromatograms (XIC) where the presence of the compound in the sample leads to the corresponding chromatographic peak. The high resolving power of TOF MS allows using narrow-window XIC (normally 10 – 20 mDa width), which leads to improved selectivity and sensitivity.

2. Non-target screening – where compounds eluting from the chromatographic column may be detected and subsequently identified without any kind of previous selection. Here, the analyst is searching for actual unknown compounds, as no previous information about the compounds to be investigated is taken into account.

Identification of non-target compounds in environmental samples is an interesting feature for analytical chemists, but it is still a challenge. Its routine application in environmental laboratories will take some time, as the possibilities of success when identifying unknown compounds at low levels in environmental complex matrices are still very scarce (Ibáñez et al. 2005, 2008). Thus, this chapter is focused on the screening of target compounds (post-target approach), taking illicit drugs of abuse in water as an illustrative example.

The main benefit of a post-target approach is the possibility to detect (almost) an unlimited number of compounds of interest, without using reference standards or performing additional analysis. Obviously, the compounds investigated should fit the requirements of sample preparation and MS analysis to be able of being detected. The accurate mass spectra can rapidly and simply be evaluated using an in-house compound database and powerful software, currently available, such as ChromaLynx XS (Waters). In subsequent steps, in order to facilitate a wider screening, earlier data can be reprocessed and reevaluated using new or extended databases.

In this chapter, the potential of QTOF MS combined with ultrahigh-pressure liquid chromatography (UHPLC) for screening and confirmation of illicit drugs in wastewater is illustrated. The work is focused on the applicability of the post-target approach, which, from the authors' point of view, is the most relevant application when using TOF in environmental MS.

4.2. Analytical strategies for the determination of illicit drugs in the environment

The occurrence of emerging contaminants in the environment has been a major issue for analytical chemists during the last few years (Richardson 2008, 2009). Emerging environmental contaminants, such as pharmaceuticals, personal care products, and veterinary drugs received much interest (Barceló and Petrovic 2007) and studies of illicit drugs increased, since their presence were reported in surface water and urban wastewaters (Jones-Lepp et al. 2004; Zuccato et al. 2005; Castiglioni et al. 2006). The low concentration levels of illicit drugs typically found in the aquatic environment in combination with the complexity of the matrix make their reliable determination difficult. Therefore, highly selective and sensitive analytical methods are required.

Several multiclass methods based on liquid chromatography (LC) coupled to triple quadrupole (QqQ) instruments have been developed for quantification of illicit drugs in environmental samples (Castiglioni et al. 2008). Until now, most of the methods developed have used off-line solid-phase extraction (SPE) for sample pretreatment and preconcentration. Typically, deuterated analytes are used as surrogate internal

standards to compensate for possible errors resulting from matrix effects, as well as those associated with the sample preparation step. The use of QqQ analyzers in selective-reaction monitoring (SRM) mode allows the minimization, or, in some cases, the elimination, of interferences thereby improving selectivity and sensitivity because of the possibility of adequate precursor and product ion selection, leading to low chemical noise in the chromatograms. The excellent sensitivity together with the proved robustness of this analyzer make of LC-MS/MS with triple quadrupole a powerful analytical tool for quantification purposes of illicit drugs at the low concentration levels normally present in water. In spite of the good selectivity of tandem MS, when using low-resolution instruments, such as QqQ, at least two SRM transitions should be monitored for a safe identification, but the quality of the transitions should be taken into account, avoiding nonspecific transitions (e.g. H_2O, CO_2, HCl) in order to prevent false positives or even false negatives (Hernandez et al. 2005a, 2005b; Pozo et al. 2006). Thus, when acquiring at least two transitions, together with the accomplishment of the ion ratios deviations between recorded transitions, QqQ analyzers in SRM mode are highly reliable and suitable for trace determination of drugs of abuse in water (Bijlsma et al. 2009).

As any analytical technique, LC-MS/MS with QqQ analyzer also has some limitations. The number of compounds to be included in the method is restricted, since only a limited number of transitions may be selected. Including many compounds in a multi-residue method may lead to a loss in sensitivity, as an increase in acquired transitions usually involves a decrease in either the time of acquiring the transition (dwell time) or the number of points obtained per peak. Thus, a compromise between sensitivity and peak shape has to be reached when developing multiresidue methods. However, recent developments in triple quadrupole instruments, such as dynamic or scheduled-SRMs, permits the counterbalancing of this limitation. Another issue is that reference standards of each target compound are needed for SRM optimization. Most of the illicit drugs standards are expensive and it is often time-consuming to purchase them, because of extensive administrative requirements imposed on the importation or exportation of controlled substances. However, the main limitation is that other nonselected compounds cannot be revealed, even if they are present at high levels in

the sample, since the analytes are preselected before MS data acquisition. This might be considered as an important drawback, especially when a wide-scope screening is the objective of analyses.

The low sensitivity in full-scan mode, the measurements in nominal mass, and need of reference standards for identification make it unadvisable to use QqQ instruments in post-target or non-target screening of drugs of abuse in wastewater. Conversely, TOF MS instruments have shown a great potential for screening and confirmation purposes in various fields. Compounds such as pesticides, pharmaceuticals and antibiotics have been screened in environmental samples by LC-TOF MS (Petrovic et al. 2006; Ibáñez et al. 2008, 2009). Besides, several studies have been reported on screening of compounds of toxicological interest (including illicit drugs) in forensic samples (Pelander et al. 2003; Ojanperä et al. 2006; Badoud et al. 2009), and on screening of pesticides in food (Ferrer and Thurman 2007; García-Reyes et al. 2008; Grimalt et al. 2009). Several of these methods are directed towards a relatively high number of compounds (around or even more than 100) exploiting the elevated resolution and mass-accuracy capabilities of TOF analyzers.

Because of the novelty of this approach, a wide-scope screening (300 – 400 analytes) has only been applied to a few studies (Grimalt et al. 2009; Mezcua et al. 2009). The ability of TOF MS to provide a notable amount of relevant chemical information in a single experiment makes this technique very attractive for a wide-scope screening of illicit drugs in water using a post-target style.

4.3. Screening by UHPLC-QTOF MS

4.3.1. Methodology

As earlier stated, sensitive full spectrum data acquisition, high-mass resolution, speed, and mass accuracy of new generation TOF MS or QTOF MS instruments allows a highly reliable confirmation and a wide-scope screening of illicit drugs in the aquatic environment.

Orthogonal acceleration TOF MS instruments permit measurements with a mass accuracy commonly better than 5 ppm and for mass resolution exceeding 10,000 full width half maximum (FWHM). The mass of any ionizable component in different sample matrices can be measured accurately, which gives high confidence to the identification process, and also implies the possibility to distinguish in between isobaric compounds. Isobars have identical nominal mass, but different elemental composition and thus different exact mass. When using QqQ instruments (which work in nominal mass), isobars can be discriminated from their retention time or fragmentation. Thus, when chromatographic separation is not fully achieved, a false positive or negative (non-compliance of ion ratio) might be reported. TOF MS allows discriminating isobaric compounds from differences in mass defect reducing the possibilities of false identification. Moreover, the elevated mass resolution of modern TOF analyzers allows obtaining narrow-window XIC. For example, a 0.02 Da mass window can be used at the exact mass of the selected compound. Decreasing the mass window leads to a reduction of interferences from isobaric compounds and an increase of the signal-to-noise ratio (Hogenboom et al. 1999; Petrovic et al. 2006; Sancho et al. 2006; Ibáñez et al. 2008) with the final result of much improved selectivity. As an example, in the analysis of a wastewater sample we observed two chromatographic peaks, corresponding to the antibiotics oxonilic acid (*m/z* 262.0715) and flumequine (*m/z* 262.0879), when a mass window of 1 Da was selected. Reducing the mass window from 1 to 0.02 Da led to only one peak per window, which corresponded to each individual antibiotic (Ibáñez et al. 2009).

An interesting feature in order to generate molecular formulas and to facilitate an extra confident identification is the use of different isotopic filters. These filters work based on the isotopic pattern deviation between the empirically measured and the theoretical spectrum. The presence of an abundant isotopic pattern in the analyte molecule helps to confirm the presence of that compound in the sample. Thus, the presence of atoms such as carbon, chlorine, bromine, or sulphur in the molecule gives a characteristic isotopic pattern that allows reducing the number of possible elemental compositions for a certain mass-accuracy window. The match between empirical and theoretical data is given by the isotope fit (i-FIT) or SigmaFIT values. These values are calculated, taking

into account not only the isotopic distribution but also the accurate masses. The lower the value, the more plausible the elemental composition (Ojanperä et al. 2006; Ibáñez et al. 2008).

One of the main drawbacks of TOF or QTOF instruments compared to QqQ working in SRM mode is their lower sensitivity, since the principal difficulties for the analysis of illicit drugs in wastewater and surface water are their low concentration levels in combination with the complexity of the matrix. This disadvantage might be resolved, or at least minimized, by activating specific functions like, for example, the enhanced duty cycle (EDC) mode in the instrument used in our work (QTOF Premier, Waters). When TOF operates in EDC mode, the ion abundance is expected to be improved, as ions are transported in packets which make it possible to synchronize the TOF pusher with each ion packet and enhance the duty cycle over a selected *m/z* range (Weaver et al. 2007).

On the contrary, in some occasions (e.g. during musical events) concentrations of illicit drugs in urban wastewater are higher (Bijlsma et al. 2009). This might provoke detector saturation and mass shifts that would increase mass errors, negatively affecting the identification of potential positives. Dynamic-range enhancement (DRE) is an approach that not only affects the detector saturation, but also the accuracy of mass measurements. The effectiveness of DRE has been reported in the bibliography (Weaver et al. 2007; Tiller et al. 2008; Ibáñez et al. 2009) leading to significant improvement in quantitative and qualitative analysis (i.e., to determine the accurate mass of compounds with more certainty over a large range of concentrations).

In the last few years, several methods using ultra-high-pressure liquid chromatography (UHPLC) coupled to different MS/MS analyzers have been reported for screening of illicit drugs in water (Huerta-Fontela et al. 2007; Kasprzyk-Hordern et al. 2007; Bijlsma et al. 2009). Because of the high selectivity of tandem MS, the importance of an efficient chromatographic separation might be neglected. However, efficient chromatography is essential to avoid or minimize matrix effects, especially in trace analysis, where failure to completely separate analytes from each other and from the matrix components

may result in reporting false positives or negatives (Niessen et al. 2006; Petrovic et al. 2006; Pozo et al. 2006; Bijlsma et al. 2009). UHPLC presents several advantages over conventional liquid chromatography as it generates narrow peaks (increasing peak height and improving sensitivity), facilitates resolving the analytes and matrix interferences (a crucial aspect when dealing with very complex samples), and allows multiresidue analysis to be performed with shorter chromatographic runs. Because of the better resolution and ultra-fast separation, UHPLC has become a powerful tool with significant improvement in terms of sample throughput and sensitivity. However, it requires a fast detector, adequate for narrow chromatographic peaks. Triple quadrupole instruments rely on defined dwell times to monitor analytes. This is not a limitation when a few analytes are monitored; however, it may become a potential problem for multiresidue methods where a wide-scope screening is the objective. Although modern QqQ instruments have been significantly improved, the shortening of dwell times negatively affects the sensitivity and reproducibility. TOF, being inherently faster than QqQ, might detect a huge number of analytes without compromising sensitivity and selectivity. In addition, the higher chromatographic resolution renders high-mass spectra purity. The high acquisition speed of TOF analyzers makes them fully compatible with UHPLC, which is highly beneficial for multiresidue screening methods where difficult matrices are involved (Kaufmann 2009). As an example, UHPLC-TOF MS has been successfully applied in our group for screening of antibiotics in water (Ibáñez et al. 2009).

When screening a sample for unknown, nonselected, compounds (nontarget screening), it becomes difficult to pick out individual ions, especially when the matrix is complicated or when the concentration of the compound is low. Under these circumstances, it is necessary to use powerful software with chromatographic peak deconvolution capabilities to identify the presence of multiple components and to produce pure spectra for each individual component. As the objective of this chapter is searching for illicit drugs, a more realistic and efficient approach using TOF would be the post-target screening, acquiring the full-scan spectrum at high resolution and performing subsequent nw-XIC at selected accurate *m*/z. Nevertheless, this procedure is highly time-consuming, since each exact *m*/z needs to be typed in individually.

Powerful software is currently available, which offers the possibility of applying a post-target approach using compound databases based on preselected exact masses. This permits a rapid and simple reviewing by cataloguing analytes (e.g., based on colors) as a function of mass error. In addition, this software allows simultaneously visualizing the complete mass spectrum of the positive findings. In many cases, accurate mass spectra can confirm or deny the presence of a specific compound. In theory, an unlimited number of compounds can be included in a database and expensive reference standards are not compulsory, allowing the possibility of setting up a wide-scope screening (Laks et al. 2004). However, reference standards are highly valuable, as experimental data obtained from their analysis (e.g., retention time and fragmentation) provide additional confidence in the identification process.

A database does not necessarily consist of merely illicit drugs. In fact, it is desirable to add several drug-related compounds, such as metabolites and conjugates (e.g. sulfates or glucuronides), as they might produce similar or even higher ecotoxicological problems. In addition, the occurrence of these compounds in the environment is often higher than that of parent compounds. For example, higher concentrations were found for benzoylecgonine in comparison to its parent compound, cocaine (Zuccato et al. 2005; Huerta-Fontela et al. 2007; Gheorghe et al. 2008; Bijlsma et al. 2009). In certain occasions, it can also be very interesting to include specific fragment ions in the database.

The compromised standard conditions for a wide-scope screening might lead to extensive in-source fragmentation for certain unstable compounds, and only searching for (de)protonated molecules might be insufficient. Moreover, today´s medicines can be tomorrow´s drugs of abuse, as some prescription drugs have the potential of abuse and the inventiveness of drug addicts and dealers are high. In other words "new" drugs of abuse may be "discovered" and are of interest in a later stage. Since evaluation of TOF MS data is performed after acquisition, data can be reexamined in searching for other or new compounds. By including their theoretical mass and empirical formulas into a database, the earlier obtained data can be reprocessed and reevaluated without performing additional analysis. Without the need of reanalyzing the samples,

the presence of many compounds can be investigated months or even years later, taking advantage of the abundant and useful information contained in TOF full-spectra acquisition.

As previously stated, TOF instruments normally measure the accurate mass of (de)protonated molecules, whereas QTOF MS can provide additional structural information by obtaining full spectra of products ions at accurate mass. QTOF MS is an excellent technique to perform valuable MS/MS experiments for elucidation purposes. The accurate mass together with the acquisition of the full product ion spectrum is also a powerful tool for the unequivocal confirmation of positives in target analysis. In particular, for a number of compounds (e.g., isomers), information on products ions is almost indispensable for a correct identification. Isomers share the same empirical formulas, and, therefore, their exact mass, and can only be discriminated either from retention times or from fragmentation pathway. For example, thanks to the use of QTOF MS/MS experiments, the hydroxyl metabolite of the insecticide buprofezin could be distinguished from the sulfoxide metabolite in a banana sample that contained the parent pesticide (Grimalt et al. 2007). Both metabolites share the same empirical formula and exact mass, but their different fragmentation when performing MS/MS experiments gave us the information required to discriminate between them and to assign the correct chemical structure to the metabolite found in the sample.

On the other hand, the potential of QTOF used in MS/MS mode for wide-scope screening purposes is limited, as this implies preselection of an analyte *m/z* in order to filter it in the quadrupole. However, information of both (de)protonated molecule and fragment ions from a single experiment is feasible by QTOF MS running in the so-called MS^E acquisition mode, without the need of selecting the precursor ion or losses of isotopic information. Obviously, a QTOF instrument can be used in a TOF mode, which would be the approach recommended for wide-scope screening purposes. However, this tandem mass analyzer offers the possibility of promoting fragmentation in the collision cell, when also being used as a TOF. MS^E experiments involve the simultaneous acquisition of exact TOF MS data at low- and high-collision energy (Castro-Perez et al. 2002; Plumb et al. 2006; Weaver et al. 2007; Tiller et al. 2008). By applying low energy (LE)

in the collision cell, compound fragmentation is minimized; consequently, information given in the accurate TOF MS spectra corresponds mainly to nonfragmented ions. At high collision energy (HE), a more efficient fragmentation will take place, resulting in more abundant accurate mass fragments, but still maintaining, in most of cases, the (de)protonated molecule information. Although these ions are not generated in a traditional MS/MS manner, analog terms are sometimes used because of similarities in both approaches by using the same QTOF instrument. Thus, spectra at low energy are mainly dominated by "precursor" ions, while high-energy spectra are dominated by "product" ions. Although similar information might be acquired by varying the cone voltage of a single TOF MS, to produce in-source fragmentation, data obtained are of reduced signal intensity, because of the formation of adducts and the effect of neutrals in the source (Plumb et al. 2006). In addition, we also observed that mass accuracy deteriorated and less abundant fragmentation was obtained in comparison to the use of a collision cell (Díaz et al. 2009).

Hence, the MS^E approach applied in QTOF instruments to produce collision cell fragmentation is preferable. All MS data obtained by this approach are processed after acquisition, allowing the search for many other compounds, without additional analysis, simply by reprocessing the sample data. Despite these advantages, the technique can be acknowledged as pseudo-MS/MS, the main drawback of MS^E. Whilst the first (quadrupole) mass analyzer operates in wide band transition mode, multiple components might simultaneously enter the mass spectrometer, making the interpretation of the high-energy mass spectra difficult. For this reason, good chromatographic separation is paramount. When working with UHPLC-QTOF MS in MS^E acquisition mode, full advantage is taken of the higher chromatographic resolution of UHPLC and both (de)protonated molecule and fragmentation data can rapidly be obtained, facilitating simultaneous screening and confirmation.

4.3.2. Application to real samples

To illustrate the potential of QTOF MS for confirmation of illicit drugs in the aquatic environment, we will show illustrative examples taken from effluent urban wastewater samples (EWW) of the Castellón province (Spain), which were analyzed along 2008-

2009. These complex matrix samples were firstly analyzed by a target UHPLC-QqQ-MS/MS method developed for eleven drugs and metabolites (Bijlsma et al. 2009). Most of samples analyzed contained benzoylecgonine, a major cocaine metabolite. It is interesting to note that another cocaine metabolite, norcocaine, shares the same empirical formula, and thus the same exact mass ($[M+H]^+$, *m*/z 290.1392). As reference standards were available to the authors and chromatographic separation was satisfactory, discrimination of both analytes could be made from their retention times (benzoylecgonine, 4.99 min. and norcocaine, 5.37 min.). However, reference standards were not indispensable when analysis was performed by QTOF MS, as these compounds could also be discriminated from fragmentation (**Figure 4.1**).

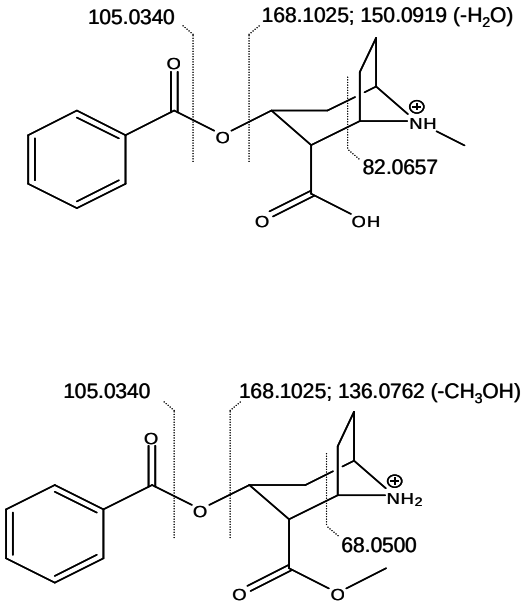

Figure 4.1. Top, fragmentation pattern benzoylecgonine $[M+H]^+$. Bottom, fragmentation pattern norcocaine $[M+H]^+$.

Although good chromatographic separation is important, these two isomers could be discriminated by specific fragment ions. This is illustrated in **Figure 4.2**, where full-scan spectra of an EWW sample (**Figure 4.2a**) and benzoylecgonine and norcocaine standard (**Figure 4.2b and 4.2c**, respectively) by UHPLC-QTOF MS running in both MS^E (LE

and HE) and MS/MS acquisition mode are depicted. The relative intensities and accurate masses of the main ions were compared with those of a reference standard. For almost all ions, experimental accurate masses compared to theoretical exact masses (**Figure 4.1**) presented errors lower than 2 mDa, giving a high degree of confidence for confirmation of benzoylecgonine. Although relative intensities of high-energy MS^E spectra versus MS/MS spectra are slightly different, because of a small change in the collision energy-ramps applied to the protonated molecule, spectra were comparable, demonstrating similar capabilities for both approaches. Nevertheless, QTOF MS/MS provides better spectra with higher sensitivity, as it is more efficient because of the improved duty cycle as well as to cone voltage optimization.

Similarly, QTOF MS allowed confirming positives found by LC-MS/MS QqQ, thanks to the accurate mass measurements of the (de)protonated molecule and the most abundant fragments at LE and HE simultaneous acquisition. As an example, **Table 4.1** shows the drugs of abuse confirmed by MS^E in an effluent wastewater sample.

Table 4.1. Confirmation of Different Positives of an Effluent Wastewater, in MS^E Acquisition Mode at Low (LE) and High (HE) Energy.

Compound	Precursor/ Fragment	Exact mass	Accurate mass	Error (mDa)
MDMA	$C_{11}H_{16}NO_2$ (LE)	194.1181	194.1164	-1.7
	$C_{10}H_{11}O_2$	163.0759	163.0775	1.6
	$C_8H_7O_2$	135.0446	135.0441	0.2
	C_9H_9O	133.0653	133.0661	-0.8
Cocaine	$C_{17}H_{22}NO_4$ (LE)	304.1549	304.1562	1.3
	$C_{10}H_{16}NO_2$	182.1181	182.1178	-0.3
	$C_9H_{12}NO$	150.0919	150.0923	0.4
	C_5H_8N	82.0657	82.0648	0.9
Benzoylecgonine	$C_{16}H_{20}NO_4$ (LE)	290.1392	290.1407	1.5
	$C_9H_{14}NO_2$	168.1025	168.1025	0
	$C_9H_{12}NO$	150.0919	150.0918	-0.1
	C_7H_5O	105.0340	105.0351	1.1
Norbenzoyl-ecgonine	$C_{15}H_{18}NO_4$ (LE)	276.1236	276.1260	2.4
	$C_8H_{12}NO_2$	154.0868	154.0882	1.4
	$C_8H_{10}NO$	136.0762	136.0741	-2.1
	C_7H_5O	105.0340	105.0336	-0.4

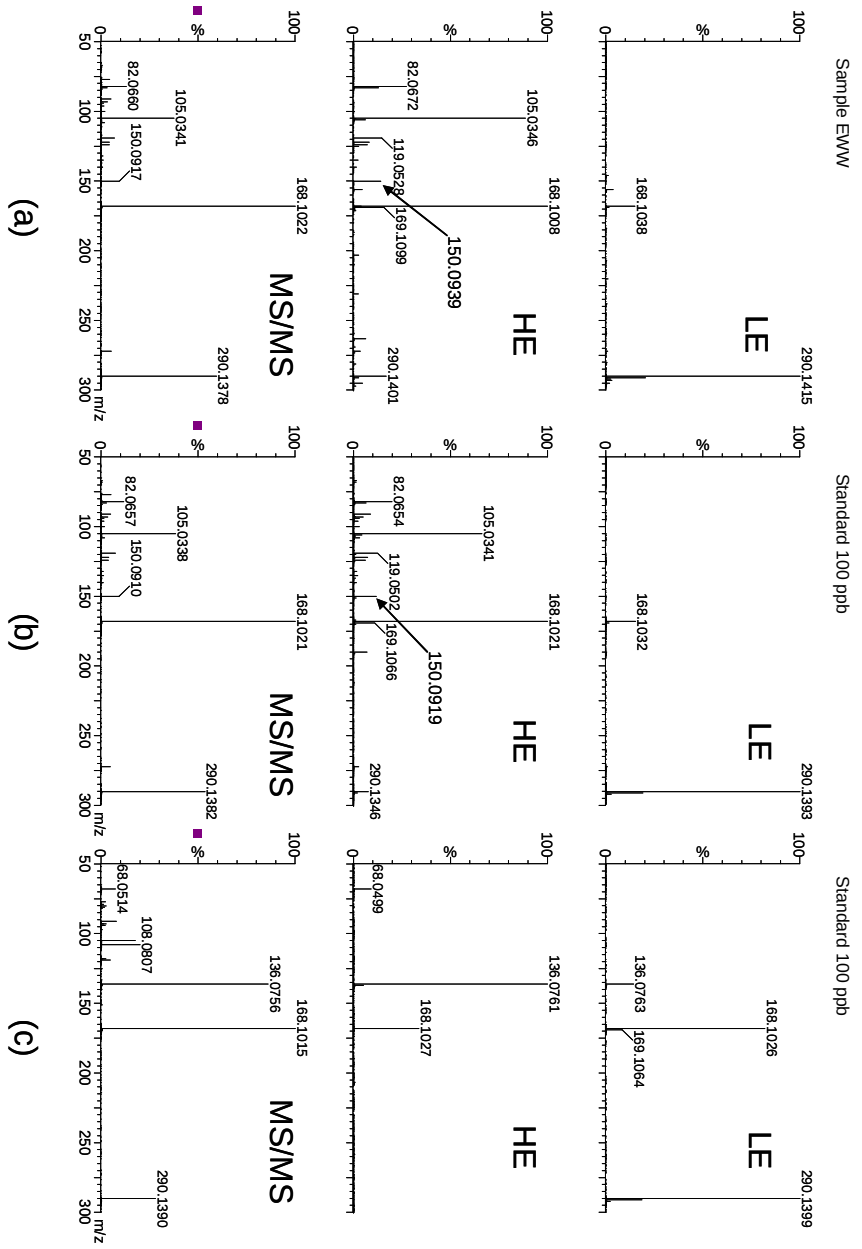

Figure 4.2. Confirmation of Benzoylecgonine (BE) in effluent wastewater (EWW). Full-scan spectra obtained by UHPLC-QTOF MS running in MS^E (low-collision energy LE, high-collision energy HE) and MS/MS acquisition mode (a) EWW sample (b) BE standard (c) norcocaine standard.

In addition to the reliable confirmation of compounds identity, QTOF MS in MS^E acquisition mode is an excellent tool for a wide-scope screening of illicit drugs in a post-target way. Reference standards are not fully necessary and searching of compounds can be easily extended, permitting a wider screening. In our case, a specific software (ChromaLynx XS, Waters) allowed a rapid and simple reviewing by cataloguing, based on color, the accurate mass of the analyte protonated molecule $[M+H]^+$, as a function of mass error. In this way, an effluent wastewater sample was suspected to contain ketamine (**Table 4.2**). This compound had not been considered in the list of target analytes included in the LC-MS/MS QqQ method applied in our previous work, but it was subsequently investigated by reexamining data obtained by LC-QTOF MS.

Table 4.2. Ketamine and Codeine Discovered after QTOF Screening of an Effluent Wastewater by MS^E Acquisition Mode at Low (LE) and High (HE) Energy.

Compound	Precursor/ Fragment	Exact mass	Accurate mass	Error (mDa)
Ketamine	$C_{13}H_{17}ClNO$ (LE)	238.0999	238.1017	1.8
	C_7H_6Cl	125.0159	125.0166	0.7
Codeine	$C_{18}H_{22}NO_3$ (LE)	300.1600	300.1610	1.0
	$C_{13}H_9O$	181.0653	181.0636	1.7
	$C_{13}H_9$	165.0704	165.0731	2.7
	$C_{12}H_8$	152.0626	152.0641	1.5

When performing a narrow-window XIC at its exact mass, an abundant peak was observed (**Figure 4.3a**). The MS^E spectra at LE and HE are shown in **Figure 4.3b**. Notice from the LE spectra that a characteristic isotopic pattern, corresponding to ^{35}Cl and ^{37}Cl, can be observed. As stated before, the quadrupole operates during MS^E acquisition in wide-band transition mode in such a way that all ions are transmitted into the collision cell, providing useful information regarding isotopic pattern. Ketamine ($[M+H]^+$, *m*/z 238.0999) contains one chlorine atom, which is in agreement with the isotopic pattern mentioned above. In addition, *m*/z 207.0596 fragment could be linked to *m*/z 238.1017 (a loss of $-CH_5N$); since there is no loss of chlorine, the isotopic peak (*m*/z 209.0572) corresponding to ^{37}Cl was present at around 30% of that of ^{35}Cl, as expected (**Figure 4.3b**, top). Another fragment, *m*/z 125.0173, also show a small peak at *m*/z 127.0133, however the ratio does not fit with that expected for one Cl atom. At HE, complete fragmentation towards *m*/z 125.0166 seems to occur, showing neither other

fragment nor isotopic peak. Despite this fact, we did suspect this fragment to contain chlorine, because of the mass error of the fragment in combination with the few possibilities of ketamine to have a product ion at *m/z* 125 without Cl. The disappearance of the isotopic peak might be explained by the fact that the background noise was subtracted from the combined peak spectra to get cleaner spectra. To ensure the compound identity, ketamine reference standard was purchased and MS/MS experiments for both reference standard and sample were carried out (**Figure 4.3c**). This allowed us to confirm the presence of ketamine in the sample, as at least five product ions were coincident with low mass errors.

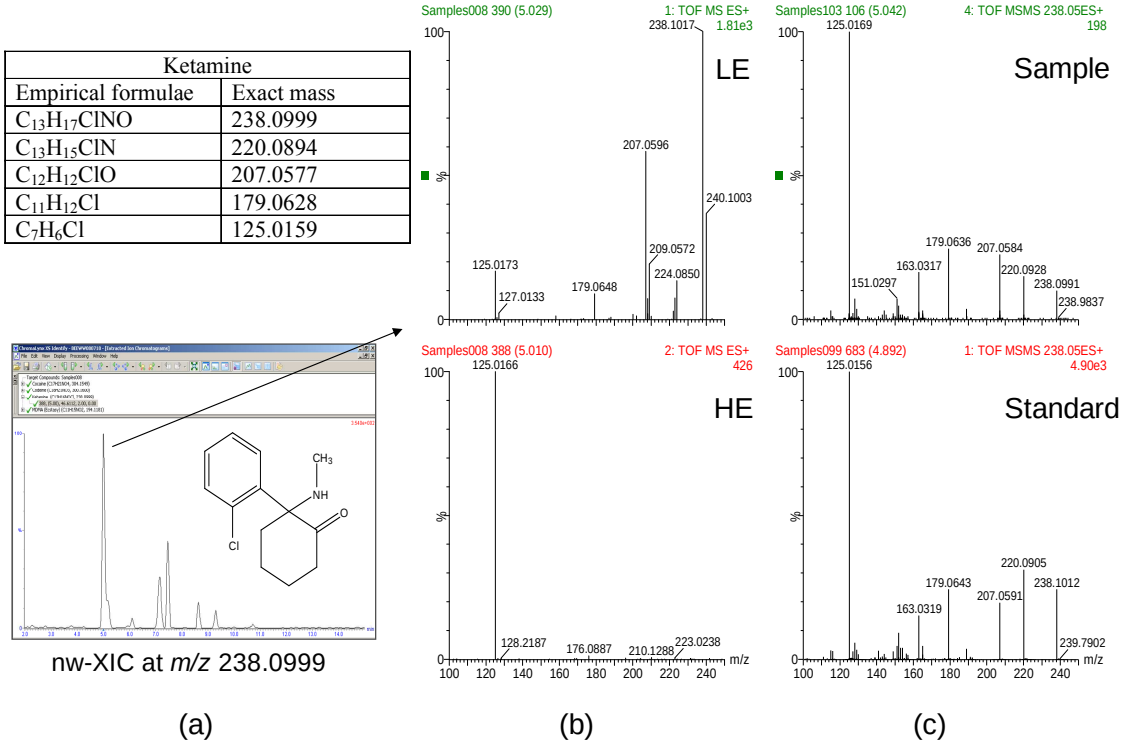

Figure 4.3. (a) nw-XIC at *m/z* 238.0999 of an effluent wastewater sample. Table: exact mass of ketamine and fragments; (b) full-scan MS^E spectra of sample peak (Rt time: 5.0 min); (c) full-scan MS/MS spectra of the sample (top) and ketamine standard (bottom).

In addition, by using the reference standard, the identity of the analyte could also be confirmed by retention time and semiquantification could even be performed.

Another example of drugs of abuse found in the samples analysed by MS^E approach was codeine (see **Table 4.2**), also discovered in an effluent wastewater sample.

The illustrative examples described here show the great potential of UHPLC-QTOF MS in MS^E acquisition mode, for (post-target) wide-scope screening and confirmation of illicit drugs in complex environmental samples.

4.4. Conclusions

Most of the existing screening methods for illicit drugs in environmental matrices are focussed on (pre)target analysis, typically using LC-MS/MS with triple quadrupole analyzers in SRM mode. These methods require a reference standard of each target compound for optimization, compound identification, and quantification. This approach is highly suitable and reliable for trace determination of a limited number of compounds. However nonpreselected compounds that might be present in samples, even at high concentrations, would not be revealed using this approach. Depending on the number of transitions monitored and on their specificity, additional confirmation is sometimes necessary in order to prevent false positives or negatives.

Other strategies would be highly useful to apply a wide-scope screening for other compounds of interest. LC-QTOF in MS^E acquisition mode is much more appropriate for this goal as most of drugs of abuse and their metabolites are LC-amenable, and QTOF offers useful relevant information for a reliable identification of compounds detected in samples. By means of specialized software and appropriate compound database, accurate mass information can be processed after MS full acquisition using mass error and retention times, when available. This approach allows a safe identification of the compound detected, because of accurate mass data and fragmentation, and is highly useful for simultaneous rapid wide-scope screening and confirmation of illicit drugs and their metabolites. Since screening and confirmation is performed after MS acquisition, data can be reprocessed and reevaluated using new or modified databases in search for other interesting compounds. In theory, a huge number of compounds could be detected and confirmed, without performing additional analysis and without the need of reference standards. Obviously, compounds subjected to investigation have to satisfy the requirements for LC-MS analysis (e.g., they have to be ionized in the commonly API sources, mostly ESI) and they have to be compatible with the sample treatment applied. Therefore, for a wide-scope screening minimum sample treatment would be desirable in order to extend the scope of the method to as many compounds as possible. New generations of QTOF instruments, with improved resolution and sensitivity, will facilitate direct injection of aqueous samples (in some cases after

centrifugation or acidification) for detection of drugs present at sub-part per billion levels.

Although negative ionisation was not taken into consideration in our work, because of the basic character of most illicit drugs, this acquisition mode would complete an even wider scope screening. In addition, unknown compounds, like some metabolites or transformation products (TPs), might also be revealed, by applying a nontarget search using the same accurate mass data and other relevant information given by QTOF MS analysis (e.g. isotopic distribution, fragment ions) together with appropriate databases.

UHPLC-QTOF MS has been successfully applied to urban wastewater in order to investigate the presence of illicit drugs. Previous positives of MDMA, cocaine, benzoylecgonine and norbenzoylecgonine found by triple quadrupole LC-MS/MS have been confirmed and other nonpreselected compounds, like ketamine or codeine, have been detected by reexamining MS data after MS^E acquisitions.

The strategy described in this chapter for illicit drugs can also be applied to many other contaminants that could be present in the aquatic environment like antibiotics, pesticides and transformation products, widening the searching of organic pollutants up to more than 1000 compounds. This illustrates the strong potential of UHPLC-QTOF MS for screening purposes.

References

Badoud F, Grata E, Perrenoud L, Avois L, Saugy M, Rudaz S, Veuthey J-L (2009). Fast analysis of doping agents in urine by ultra-high-pressure liquid chromatography-quadrupole time-of-flight mass spectrometry I. Screening analysis. Journal of Chromatography A 1216:4423 - 4433.

Barceló D, Petrovic M (2007). Pharmaceuticals and personal care products (PPCPs) in the environment. Analytical and Bioanalytical Chemistry 387:1141 - 1142.

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

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

Castiglioni S, Zuccato E, Chiabrando C, Fanelli R, Bagnati R (2008). Mass spectrometric analysis of illicit drugs in wastewater and surface water. Mass Spectrometry Reviews 27:378 - 394.

Castro-Perez J, Hoyes J, Major H, Preece S (2002). Advances in MS-based approaches for drug and metabolism studies. Chromatographia 55:S59 – S63.

Díaz R, Ibáñez M, Sancho JV, Hernández F (2009). Investigation of the advanced functionalities of a new generation quadrupole time-of-flight mass analyzer. Application to the creation of empirical mass spectra library for screening of organic pollutants. Rapid Communications in Mass Spectrometry, under revision.

Ferrer I, Thurman EM (2007). Multi-residue method for the analysis of 101 pesticides and their degradates in food and water samples by liquid chromatography/time-of-flight mass spectrometry. Journal of Chromatography A 1175:24-37.

García-Reyes JF, Gilbert-López B, Molina-Díaz, A, Fernández-Alba AR (2008). Determination of pesticide residues in fruit-based soft drinks. Analytical Chemistry 80:8966-8974.

Gheorghe A, van Nuijs A, Pecceu B, Bervoets L, Jorens PG, Blust R, Neels H, Covaci A (2008). Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry. Analytical and Bioanalytical Chemistry 391:1309-1319.

Grimalt S, Pozo, OJ, Sancho JV, Hernández, F (2007). Use of liquid chromatography coupled to quadrupole time-of-flight mass spectrometry to investigate pesticide residues in fruits. Analytical Chemistry 79:2833-2843.

Grimalt S, Sancho JV, Pozo OJ, Hernández F (2010). Quantification, confirmation and screening capability of UHPLC coupled to triple quadrupole and hybrid quadrupole time-of-flight mass spectrometry in pesticide residue analysis. Journal of Mass Spectrometry 45, 421-436.

Hernández F, Pozo OJ, Sancho JV, López FJ, Marín JM, Ibáñez M (2005a). Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS^2 using triple quarupole and hybrid quadrupole time-of-flight analyzers. Trends in Analytical Chemistry, 24:596-612.

Hernández F, Sancho JV, Pozo OJ (2005b). Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples. Analytical and Bioanalytical Chemistry 382:934 - 946.

Hogenboom AC, Niessen WMA, Little D, Brinkman UATh (1999). Accurate mass determinations for the confirmation and identification of organic microcontaminants in surface water using on-line solid-phase extraction liquid chromatography electrospray orthogonal-acceleration time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 13:125 – 133.

Huerta-Fontela M, Galceran MT, Ventura F (2007). Ultraperformance liquid chromatography-tandem mass spectrometry analysis of stimulatory drugs of abuse in wastewater and surface waters. Analytical Chemistry 79:3821 – 3829.

Ibáñez M, Sancho JV, Pozo OJ, Niessen W, Hernández F (2005). Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water. Rapid Communications in Mass Spectrometry, 19:169-178.

Ibáñez M, Sancho JV, McMillan D, Rao R, Hernández F (2008). Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-flight mass spectrometry. Trends in Analytical Chemistry 27(5):481 - 489.

Ibáñez M, Guerrero C, Sancho JV, Hernández F (2009). Screening of antibiotics in surface and wastewater samples by ultra-high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry. Journal of Chromatography A 1216:2529 - 2539.

Jones-Lepp TL, Alvarez DA, Petty JD, Huckins JN (2004). Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents. Archives of Environmental Contamination and Toxicology 47: 427-439.

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ (2007). Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra performance liquid chromatography-positive electrospray ionisation tandem mass spectrometry. Journal of Chromatography A 1161:132 - 145.

Kaufmann A (2009). Quantitative analysis of veterinary drug residues by sub 2-µm particulate high-perfromance liquid chromatography columns and time-of-flight mass spectrometry (UPLC-TOF). In: Ferrer I, Thurman EM, editors. Liquid chromatography time-of-flight mass spectrometry; Principles, tools, and applications for accurate mass analysis. New Jersey: Hoboken; p 133 – 150.

Laks S, Pelander A, Vuori E, Ali-Tolppa E, Sippola E, Ojanperä I (2004). Analysis of street drugs in seized material without primary reference standards. Analytical Chemistry 76:7375 – 7379.

Mezcua M, Malato O, García-Reyes JF, Molina-Díaz A, Fernández-Alba AR (2009). Accurate-mass databases for comprehensive screening of pesticide residues in food by fast liquid chromatography time-of-flight mass spectrometry. Analytical Chemistry 81:913 –929.

Niessen WMA, Manini P, Anderoli R (2006). Matrix effects in quantitative pesticide analysis using liquid chromatography-mass spectrometry. Mass Spectrometry Reviews 25:881 – 899.

Ojanperä S, Pelander A, Pelzing M, Krebs I, Vuori E, Ojanperä I (2006). Isotopic pattern and accurate mass determination in urine drug screening by liquid chromatography/time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 20: 1161 – 1167.

Pelander A, Ojanperä I, Laks S, Rasanen I, Vuori E (2003). Toxicological screening with formula-based metabolite identification by liquid chromatography/time-of-flight mass spectrometry. Analytical Chemistry 75:5710 – 5718.

Petrovic M, Gros M, Barcelo D (2006). Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. Journal of Chromatography A 1124:68 - 81.

Plumb RS, Johnson KA, Rainville P, Smith BW, Wilson ID, Castro-Perez J, Nicholson JK (2006). UPLC/MS^{E}; a new approach for generating molecular fragment information for biomarker structure elucidation. Rapid Communications in Mass Spectrometry 20: 1989 – 1994.

Pozo OJ, Sancho JV, Ibañez M, Hernandez F, Niessen WMA (2006). Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls. Trends in Analytical Chemistry 25(10):1030 - 1042.

Richardson SD (2008). Environmental mass spectrometry: emerging contaminants and current issues. Analytical Chemistry 80:4373 - 4402.

Richardson SD (2009). Water analysis: emerging contaminants and current issues. Analytical Chemistry 81:4645 - 4677.

Sancho JV, Pozo OJ, Ibañez M, Hernández F (2006). Potential of liquid chromatography/time-of-flight mass spectrometry for the determination of pesticides and transformation products in water. Analytical and Bioanalytical Chemistry 386:987 - 997.

Tiller PR, Yu S, Castro-Perez J, Fillgrove KL, Baillie TA (2008). High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line' approach for metabolite identification studies. Rapid Communications in Mass Spectrometry 22:1053 – 1061.

Weaver PJ, Laures A, Wolff J-C (2007). Investigation of the advanced functionalities of a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer. Rapid Communications in Mass Spectrometry 21:2415 – 2421.

Zuccato E, Chiabrando C, Castiglioni S, Calamari D, Bagnati R, Schiarea S, Fanelli R (2005). Cocaine in surface waters: A new evidence-based tool to monitor community drug abuse. Environmental Health: a global access science source, 4:14. Available at http://www.ehjournal.net/content/4/1/14.

Chapter 3.2.3, scientific article 5

Performance of the linear ion trap Orbitrap mass analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water

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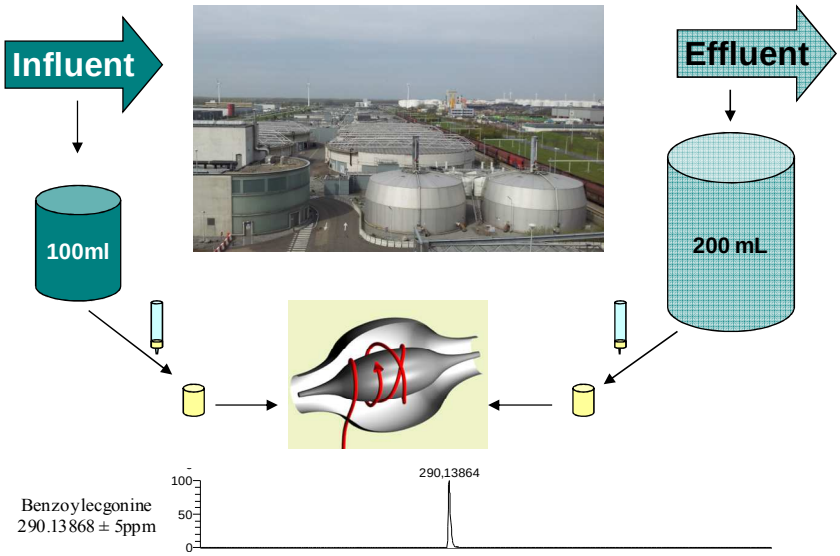
Graphical Abstract



Highlights

- A methodology was developed for the determination of 24 drugs of abuse in sewage waters
- Quantitative analyses were performed using liquid chromatography–HR Orbitrap mass spectrometer
- Compared to QqQ results, Orbitrap is almost equally sensitive
- Accurate mass full scan data allowed retrospective analysis

Abstract

This work illustrates the potential of liquid chromatography coupled to a hybrid linear ion trap Fourier Transform Orbitrap mass spectrometer for the simultaneous identification and quantification of 24 drugs of abuse and relevant metabolites in sewage water. The developed methodology consisted of automatic solid-phase extraction using Oasis HLB cartridges, chromatographic separation of the targeted drugs, full-scan accurate mass data acquisition under positive electrospray ionization mode over an *m/z* range of 50–600 Da at a resolution of 30,000 FWHM and simultaneous MS^n measurements to obtain information of fragment ions generated in the linear ion trap. Accurate mass of the protonated molecule, together with at least one nominal mass product ion and retention time allowed the confident identification of the compounds detected in these complex matrices. In addition to the highly reliable qualitative analysis, Orbitrap analyzer also proved to have satisfactory potential for quantification at sub-ppb analyte levels, a possibility that has been very little explored in the literature until now. The limits of quantification ranged from 4 to 68 ng L^{-1} in influent sewage water, and from 2 to 35 ng L^{-1} in effluent, with the exception of MDA, morphine and THC that presented higher values as a consequence of the high ionization suppression in this type of samples. Satisfactory recoveries (70–120%) and precision (<30%) for the overall procedure were obtained for all compounds with the exception of *meta*-chlorophenylpiperazine, methylphenidate and ketamine. Isotope-labelled internal standards were added to sewage samples as surrogates in order to correct for matrix effects and also for possible losses during sample treatment. The methodology developed was applied to sewage water samples from the Netherlands (influent and effluent), and the results were compared with those obtained by LC–MS/MS with triple quadrupole. Several drugs of abuse could be identified and quantified, mainly MDMA, benzoylecgonine, codeine, oxazepam and temazepam. Orbitrap also showed potential for retrospective investigation of ketamine metabolites in the samples without the need of additional analysis

Keywords

Drugs of abuse, accurate mass, orbitrap analyzer, high resolution mass spectrometry, quantitative analysis, sewage water

1. Introduction

The presence of drugs of abuse, unaltered or as metabolites, in the water cycle has spurred researchers on to investigate their occurrence in sewage water, surface water and drinking water [1 - 3]. Although concentrations found are generally low (sub μg L^{-1} level), data obtained from analysis of urban wastewaters can be used to study consumption and usage trends in communities [4]. Furthermore, environmental loads can be calculated, as their potential impact on aquatic organisms, human health and the environment may not be ruled out [1].

Most of the existing methods for determination of drugs of abuse in water are based on liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS), using triple quadrupole (QqQ) analyzers. Despite its excellent sensitivity and selectivity, this approach also has some limitations [5, 6], the main being that other drugs, different from those included in the scope of the method, may be ignored in analyses, as analyte specific information is acquired and only the target analytes are normally detected and quantified. The increasing interest of using accurate mass high resolution mass spectrometers (HRMS), e.g., Orbitrap and time-of-flight (TOF) instruments, in environmental sciences relies on its capability to perform both targeted as well as non-targeted analysis, based on full-spectrum accurate-mass acquisition at good sensitivity [7]. Efficient screening strategies using HRMS have allowed the detection and identification of various drugs of abuse in environmental and wastewater samples, in some cases even without the need of reference standards, but with high confidence due to the high mass accuracy measurements [8, 9].

Advantages of HRMS are widely recognized in qualitative analysis; however HRMS-based quantitative analyses have hardly been explored in the scientific literature until now. One of the classical criticisms concerns the relative low sensitivity and low linear dynamic range. This limitation was more evident in the first-generation instruments, e.g., first LC–TOF MS. However, the improved technology of the latest TOF instruments, i.e., higher sensitivity and resolving power, and wider linear dynamic range, provides better quantitative capabilities. This has allowed quantification of pesticides, pharmaceuticals and illicit drugs in wastewater by using LC–TOF MS [10, 11]. As for Orbitrap instruments,

good quantitative performances, i.e., high sensitivity and selectivity, have been demonstrated in some applied fields [5, 12 - 15].

Nevertheless, to the best of our knowledge, the quantitative potential of Orbitrap has not been previously demonstrated for drugs of abuse in sewage water samples. The determination of these compounds is complicated due to the complexity of the samples and low analyte concentrations. Sample pre-concentration is normally required, mostly based on solid phase extraction (SPE), but the key point is the quantification of analytes, which is problematic in LC–MS based methods due to the strong matrix effects commonly observed for this type of sample matrices. The use of isotope-labelled internal standards (ILIS) is the approach most frequently applied to solve this problem, although its application is difficult in multi-residue multiclass methods where a large number of ILIS would be required. Typically the own analyte ILIS is used, as the use of analogue compounds as internal standards does not always ensure an appropriate correction [10, 16].

In the present work, analytical methodology based on the use of SPE followed by LC coupled to a hybrid linear ion trap (LTQ) Fourier Transform (FT) Orbitrap MS, has been developed for the determination of 24 drugs of abuse and metabolites in urban wastewater. The acquisition of full-scan accurate-mass data by Orbitrap together with the simultaneous MS/MS measurements permitted by LTQ is a powerful combination for confident identification and confirmation. As the excellent qualitative potential of Orbitrap analyzer is widely accepted in the recent literature, an additional and relevant objective was to demonstrate the quantitative capabilities of this HRMS, a feature that has been little explored until now. To the best of our knowledge, the quantitative potential of Orbitrap has not been previously demonstrated for drugs of abuse in complex sewage water samples.

2. Materials and methods

2.1. Reagents

Drugs of abuse and metabolites reference standards: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylene-

dioxymethamphetamine (MDMA, or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, benzoylecgonine, heroin, morphine, 6-monoacetyl morphine (6-MAM), methadone, codeine, Δ-9-tetrahydrocannabinol (THC), 11-nor-9-carboxy-Δ-9-tetrahydrocannabinol (THC-COOH), 11-hydroxy-Δ-9-tetrahydrocannabinol (OH-THC), ketamine, methylphenidate, oxazepam, diazepam, temazepam, nordazepam, desalkyl-flurazepam, *meta*-chlorophenylpiperazine (mCPP), and fentanyl were obtained from Lipomed AG (Arlesheim, Switzerland) as solutions in methanol (MeOH), ethanol (EtOH) or acetonitrile (ACN) at a concentration of 1 g L^{-1}. Standard solutions of each compound were prepared at 36 mg L^{-1} in MeOH. A final mix solution was made by diluting aliquots from every compound individually to a concentration of 3.6 mg L^{-1}. Working mix solutions for calibration curves were made in MeOH. Before each analytical run, the calibration standards were diluted 10 times with ultrapure water resulting in a mix of water: MeOH (90:10, v/v) and were injected into the Orbitrap system. Final concentrations of standards ranged from 0.7 to 288 µg L^{-1}.

Deuterated compounds were purchased from Lipomed AG as solutions in MeOH, EtOH or ACN at a concentration of 1 g L^{-1} and were used as surrogate isotope labelled internal standards (ILIS) for quantification: amphetamine-d_{11}, methamphetamine-d_5, 3,4-methylenedioxyamphetamine-d_2 (MDA-d_2), 3,4-methylenedioxymethamphetamine-d_5 (MDMA-d_5), 3,4-methylenedioxyethyl-amphetamine-d_5 (MDEA-d_5), cocaine-d_3, benzoylecgonine-d_3, morphine-d_3, 6-monoacetyl morphine-d_3 (6-MAM-d_3), Δ^9-tetrahydrocannabinol-d_3 (THC-d_3), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol-d_3 (THC-COOH-d_3), 11-hydroxy-Δ^9-tetrahydrocannabinol-d_3 (OH-THC-d_3), oxazepam-d_5, diazepam-d_5, nordazepam-d_5. A mixed ILIS working solution was prepared in MeOH and added to all calibration standards to get a final ILIS concentration of 72 µg L^{-1}, as well as to the influent and effluent sewage water samples prior to sample treatment (final ILIS concentration in sample of 360 ng L^{-1} and 180 ng L^{-1}, respectively). All standard and working solutions were stored in amber glass bottles at −18 °C.

The ultrapure water was obtained by purifying demineralized water in a Milli-Q system from Millipore (Bedford, MA, USA). Formic acid (98–100%), HPLC-grade MeOH, EtOH and ACN were acquired from Mallinckrodt Baker (Deventer, The Netherlands).

Glass fibre filters (1 µm, type A/E) were purchased from Pall Corporation (Port Washington, NY, USA). Polyethersulfone filters (0.45 µm) with disposable setup were acquired from Nalgene (Rochester, NY, USA).

SPE cartridges, built of a hydrophilic and a lipophilic monomer (Oasis-HLB; 6 mL, 150 mg) were purchased from Waters (Milford, MA, USA).

Polytyrosine-1,3,6 standard used for mass axis calibration was purchased from Cs Bio Co. (Menlo Park, CA, USA).

2.2. Water samples

Twenty-four hours flow dependent influent and effluent composite-samples from different sewage treatment plants (STPs) located in the Netherlands were taken on the same weekend day, without accounting for lag-time. Samples were collected in amber glass bottles, and stored in the dark at 4 °C. Upon reception in the laboratory, the samples were immediately analyzed.

2.3. Extraction procedure

Prior to SPE, samples were vacuum filtered through 1 µm type A/E glass fibre filters, followed by 0.45 µm polyethersulfone (PES) filters with disposable setup. Subsequently, 200 mL of effluent sewage water, or 100 mL of influent sewage water sample, were spiked with a mixed internal standard solution to give a concentration for each compound in sample of 180 ng L^{-1} and 360 ng L^{-1}, respectively. SPE was performed automatically using a GX-274 ASPEC (Gilson). Oasis HLB cartridges were conditioned by washing and rinsing with 8 mL of ACN, 8 mL of MeOH and 8 mL of Milli-Q water. Samples were loaded onto the cartridges at 5 mL min^{-1}, and then cartridges were washed with 8 mL of Milli-Q water and dried with nitrogen for 15 min at a pressure of 1 bar. Analytes were eluted using 8 mL of MeOH at a flow of 0.5 mL min^{-1}.

The SPE eluates (MeOH) were evaporated to 200 µL at 35 °C under a gentle stream of nitrogen. Then, 250 µL of Milli-Q water was added and the remaining MeOH (200 µL) evaporated. Evaporation of the extracts was performed automatically using Barkey optocontrol (Germany). The final extract was then made up, by weight, to exactly

250 µL with Milli-Q water. As a final step, the volume was adjusted to 500 µL, by weight, with water:MeOH (80:20, v/v) to achieve a final percentage of 10% MeOH. An aliquot of the sample extract (20 µL) was injected directly into the LC-LTQ FT Orbitrap system.

2.4. Liquid chromatography

A hybrid linear ion trap Fourier Transform (LTQ FT) Orbitrap mass spectrometer was interfaced to a Surveyor HPLC system, consisting of a Surveyor auto sampler model Plus and a Surveyor quaternary gradient HPLC-pump (Thermo Fisher Scientific, Breda, The Netherlands). Chromatographic separation of the compounds was made using an XBridge C_{18} column (150 mm × 2.1 mm I.D., particle size 3.5 µm) (Waters). The pre-column used was a 4.0 mm × 2.0 mm I.D. Phenomenex Security Guard column (Bester, Amsterdam, the Netherlands). The analytical column and the guard column were maintained at a temperature of 21 °C in a column thermostat. An optimized gradient was used at a constant flow rate of 0.3 mL min^{-1} using Milli-Q water (Solvent A) and MeOH (Solvent B) both with 0.05% formic acid. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 5%; 20 min, 100%; 30 min, 100%; 32 min, 5%. Between consecutive runs, the analytical column was re-equilibrated for 10 min.

2.5. LTQ FT Orbitrap mass spectrometry

An LTQ FT Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) was used. The LTQ part of this system was equipped with an Ion Max Electrospray Ionization (ESI) probe and operated in the positive ion mode. The conditions in ESI positive mode were: source voltage 4.0 kV, heated capillary temperature 300 °C, capillary voltage 30 V and tube lens 45 V. In the LTQ component of the instrument, the temperature was set to 26 °C and helium was used as damping gas. All measurements were done using the automatic gain control (AGC) of the LTQ to adjust the number of ions entering the trap. Products ions were generated in the LTQ trap at a normalized collision energy setting of 40% and using an isolation width of 2 Da.

Full-scan accurate mass spectra (mass range from 50 to 600 Da) were obtained at a mass resolution of 30,000 FWHM (*m*/*z* 400). The total cycle time depends upon the resolution; at the selected resolution the total cycle time is about 0.55 s. The mass

spectrometer operated under data-dependent-acquisition (DDA) mode during the complete chromatographic run, in which both MS and MS^n spectra were acquired simultaneously. The instrument was initially set to operate in full-scan ('survey') mode with accurate mass measurements. When an ion exceeded a preset threshold and corresponded to the target mass list specified by the user, the instrument switched to product-ion scan mode (MS^n) in the ion-trap part with nominal mass measurements. In this way, relevant information for identification and confirmation, e.g., retention time, molecular weight and fragmentation, was obtained in a single analysis. All data were acquired and processed using Xcalibur version 2.1 software.

Mass calibration was performed with every batch run just prior to starting the batch by using flow injection of a Polytyrosine-1,3,6 solution ($[M+H]^+$ 182.01170/508.20783 and 997.39781) at a flow rate of 10 µL min^{-1}.

Identification and quantification of target compounds was performed using the accurate mass of the protonated molecule within a mass window of 5 ppm. For confirmation of the identity of the compounds, in addition to the accurate mass of the precursor ion, at least one nominal mass product ion was used together with retention time, which was compared with that of the reference standards (within 2.5%) [17, 18].

2.6. Method validation

The performance of the method was evaluated in terms of linearity, limits of quantification, trueness and precision. The overall recovery (including sample treatment and potential matrix effects) was studied and evaluated.

Instrumental linearity was estimated by analyzing standard solutions in triplicate. Satisfactory linearity was assumed when the coefficient of determination (r^2) was >0.99, based on analyte/internal standard peak areas, except for those compounds that were quantified without ILIS (absolute response).

Limit of quantification (LOQ): to facilitate the Fourier Transformation of the acquired frequency data and conversion to *m*/z in the Orbitrap, noise is filtered out. This is why the common approaches to evaluate the limits of quantification do not apply [19].

Therefore, a different approach was applied as previously reported by de Voogt et al. [20]. It is based on the matrix suppression of the deuterated analogue and the identification criteria [18] to reach enough identification points. The matrix effect (expressed as defined by Matuszewski et al. [21]) is calculated by using the area of the accurate mass signal of the deuterated standard, spiked before extraction (in matrix), divided by the average area of the deuterated standard in the calibration curve (in solvent). By using the lowest standard visible in the calibration curve which meets all the identification criteria (typically the absence/presence of the confirmation product ion is the critical parameter) and corrected for the matrix suppression and the concentration factor, the LOQ can be determined.

For those analytes for which deuterated analogues were unavailable, either the closest deuterated structure, the deuterated analyte with a similar polarity or the closest eluting compound was selected for correction. If none of the above was feasible, the LOQ was calculated based on the lowest point of the calibration line and translated to concentration in sample, taking into account the pre-concentration factors.

Trueness (estimated by means of recovery experiments) and *precision* (expressed as repeatability in terms of coefficients of variation) was evaluated by analyzing influent and effluent wastewater samples spiked at 360 ng L^{-1} and 180 ng L^{-1}, respectively.

3. Results and discussion

3.1. Sample treatment

Optimization of the extraction process and evaluation of matrix effects were made using analyte ILIS, except for some compounds of which internal standards were not available. It is expected that ILIS are affected by potential losses associated to the sample treatment and by matrix effects in the same way as the analyte [22]. An advantage of using ILIS for this evaluation is that they are not present in sewage water samples, since getting a representative genuine blank sample is one of the main difficulties for researchers working in this area.

In search for an optimum matrix effect/pre-concentration factor ratio, different volumes of wastewater sample (900 mL, 600 mL, 300 mL and 100 mL) were evaluated **Fig. 1** demonstrates the influence of intake volume on matrix suppression in a typical influent. To prevent the SPE material from clogging, samples were filtered prior to SPE through a 0.45 µm polyethersulfone filter. All experiments were performed in triplicate. A satisfactory compromise for ion suppression/enhancement versus pre-concentration factor was found by loading the cartridges with 100 mL of influent or 200 mL of effluent sewage water.

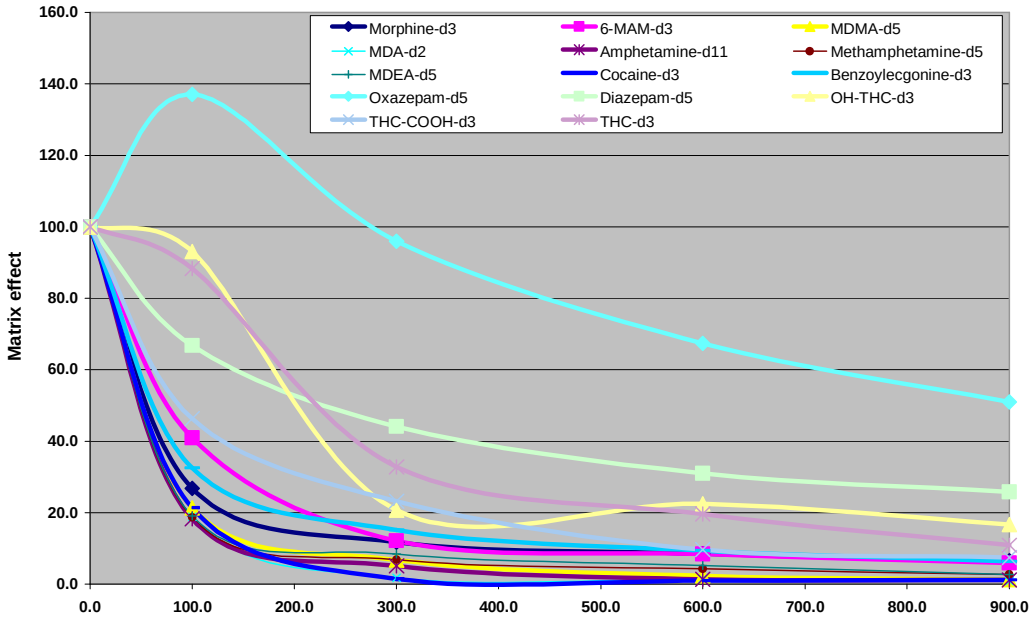


Figure 1. The influence of intake volume (100, 300, 600 and 900 mL) on the matrix effect in a typical influent.

A comparison between SPE carried out manually and automatically was made. Although recoveries were slightly lower (around 5%), automated SPE using a GX-274 ASPEC (Gilson) was preferred over the more time-consuming manual SPE.

3.2. LC-MS conditions

The most relevant parameters selected for the measurement of each analyte are shown in **Table 1**.

Table 1: Exact masses of the protonated target drugs of abuse, nominal masses and relative abundance of product ions, together with their retention times and isotope labelled internal standards used for quantification.

Compound	t_R	Precursor ion $[M+H]^+$	Product ion 1	Product ion 2		Internal standard
	(min)	*m/z*	*m/z*	*m/z*	RA (%)	
Amphetamine	10.28	136.11208	119.1	91.1	0.5	Amphetamine-d_{11}
Methamphetamine	10.64	150.12773	119.0	91.1	9.0	Methamphetamine-d_5
MDA	10.75	180.10191	163.2			MDA-d_2
MDMA	10.90	194.11755	163.1	58.0	1.0	MDMA-d_5
MDEA	11.66	208.13321	163.1	72.0	2.7	MDEA-d_5
Cocaine	13.42	304.15433	182.1	150.2	2.6	Cocaine-d_3
Benzoylecgonine	12.51	290.13868	168.2	272.2	4.8	Benzoylecgonine-d_3
Heroin	13.07	370.16490	328.2	268.2	99.1	n/a
Morphine	4.71	286.14334	201.1	229.1	51.9	Morphine-d_3
6-MAM	10.33	328.15433	211.2	268.2	73.7	6-MAM-d_3
Methadone	18.80	310.21654	265.1	247.2	0.1	n/a
Codeine	9.10	300.15942	215.2	243.1	47.7	6-MAM-d_3
THC	26.26	315.23186	259.2	193.2	76.7	THC-d_3
THC-COOH	24.84	345.20604	327.2	299.3	6.1	THC-COOH-d_3
OH-THC	24.48	331.22677	313.3			OH-THC-d_3
Ketamine	12.43	238.09932	220.1	207.1	23.9	n/a
Methylphenidate	13.61	234.14886	84.0	174.2	0.3	n/a
Oxazepam	19.53	287.05818	269.1	241.1	3.9	Oxazepam-d_5
Diazepam	20.62	285.07892	257.1	222.2	30.4	Diazepam-d_5
Temazepam	19.85	301.07383	283.0	255.2	9.2	Nordazepam-d_5
Nordazepam	20.13	271.06327	243.1	208.1	37.7	Nordazepam-d_5
Desalkyl-flurazepam	19.67	289.05385	261.1	140.0	44.5	Nordazepam-d_5
mCCP	13.62	197.08400	154.0	119.1	6.9	n/a
Fentanyl	15.66	337.22744	188.2	216.3	5.6	Nordazepam-d_5

RA: relative abundance of product ions

n/a: adequate internal standard was not available

All analytes were measured in positive mode, and the precursor ion selected was in all cases $[M+H]^+$. The instrumental configuration used in this article allows accurate mass measurement of the precursor ions in full-scan mode in the Orbitrap and, based on response thresholds, simultaneously nominal mass product ions scan in the iontrap. The exact masses of precursor ions, together with the product ions selected, measured in nominal mass, are also shown in **Table 1**. It is worth mentioning the difference with QTOF instruments where parallel acquisition cannot be performed.

Acceptable chromatographic separation of the selected drugs of abuse was achieved, with the exception of methylphenidate/mCCP, but this couple did not pose any problem for identification because of their *m*/z difference. Under the chromatographic conditions selected, the analytes retention times varied from 4.71 min (morphine) to 26.26 min (THC) (**Table 1**). A satisfactory compromise between mass resolving power and chromatographic peak shape was obtained when mass resolution was set at 30,000 FWHM with a total cycle time of about 0.55 s (including the simultaneous MS/MS acquisition in the iontrap). Accordingly, the number of data points (i.e., accurate mass scans) across each peak was at least ten and the concept of HRMS instruments (resolution ≥ 20,000 FWHM and mass accuracy ≤ 5 ppm) is maintained. The instrumental LOQs were in the 14–144 pg range (**Table 2**) except for THC and MDA (720 pg); they compare favourably to that of QTOF instruments (10–100 pg) [11] but they are considerably higher than those reported on UPLC–QqQ-MS/MS instruments while of the same order of magnitude of those achieved with a standard LC–QqQ-MS/MS system (12–530 pg) [11, 22].

Table 2: Method validation in influent (n = 4) and effluent (n = 4) sewage water.

Compound	Influent			Effluent			Linearity	
	R[a] (%)	CV[b] (%)	LOQ (ng L^{-1})	R[a] (%)	CV[b] (%)	LOQ (ng L^{-1})	r^2	Instrumental LOQ (pg)
Amphetamine	104	8	40	105	6	4	0.9960	58
Methamphetamine	98	4	15	92	7	5	0.9994	28
MDA	113	13	360[c]	92	4	158	0.9996	720
MDMA	102	4	12	97	7	4	0.9999	14
MDEA	101	4	17	98	5	4	0.9999	14
Cocaine	70	6	40	93	6	6	0.9999	14
Benzoylecgonine	111	8	10	93	7	2	0.9999	14
Heroin	70	20	19	72	21	7	0.9943	28
Morphine	102	5	360[c]	98	12	125	0.9996	144
6-MAM	117	11	19	119	15	7	0.9995	14
Methadone	73	19	45	76	5	6	0.9917	14
Codeine	90	15	19	120	26	7	0.9988	28
THC	109	11	360[c]	94	12	180[c]	0.9995	720
THC-COOH	102	7	33	90	5	7	0.9995	28
OH-THC	82	4	68	85	10	35	0.9988	58
Ketamine	48	21	10	81	7	2	0.9956	14
Methylphenidate	45	20	20	65	27	2	0.9969	14
Oxazepam	97	6	14	91	6	4	0.9994	28
Diazepam	100	4	18	94	7	6	0.9998	20
Temazepam	105	6	4	90	7	2	0.9980	28
Nordazepam	96	5	4	91	8	2	0.9995	28
Desalkyl-flurazepam	88	25	4	109	15	2	0.9994	28
mCCP	52	28	20	62	18	6	0.9911	14
Fentanyl	73	16	4	74	14	2	0.9964	14

[a] Trueness, estimated by means of recovery experiments

[b] Precision, expressed as repeatability in terms of coeficients of variation

[c] These values were derived from validation experiments (for detailed explanation see text)

3.3. Method validation

The instrumental linearity was studied by analyzing standard solutions in triplicate at nine concentration levels in the range from 0.7 to 288 µg L^{-1} (this would be equivalent to 3.6–1440 ng L^{-1} or to 1.8–720 ng L^{-1} in influent and effluent sewage water, respectively, taking into account the pre-concentration factor). The r^2 ranged satisfactorily between 0.991 and 0.999 (**Table 2**).

The matrix effects of influents and effluents were evaluated by analyzing samples from 5 different STPs. **Fig. 2** shows that in general a suppression of the signal can be observed in both influents and effluents. The matrix effects range from moderate suppression (oxazepam) to almost complete suppression (THC). Influents exhibit in general stronger suppression than effluent, despite the fact that sample intakes were twice as low.

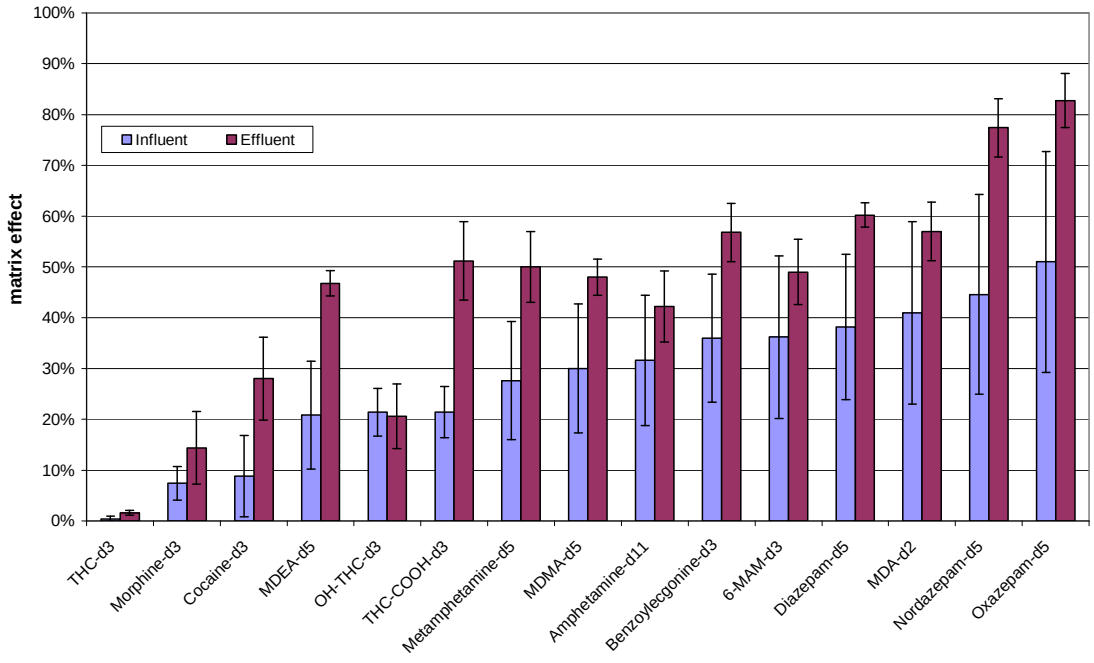


Figure 2. Average matrix effects [21] observed in both influents (100 mL) and effluents (200 mL) for 5 different STPs.

A graph showing the mass deviation of the protonated ions of selected deuterated analytes in relation to the theoretical mass over a period of 55 h is presented in **Fig. 3**. In contrast to QTOF systems [9, 11] a reference solution for continuous calibration of the mass axis was not necessary as mass accuracy remained within a deviation of 5 ppm over the whole period, illustrating the mass stability of the Orbitrap analyzer during validation and analysis.

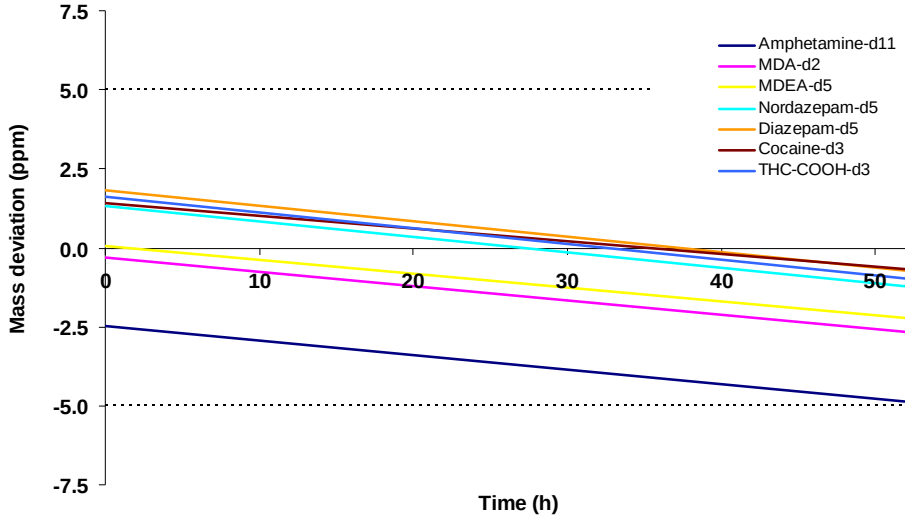


Figure 3. The mass deviation (mass drift) of the protonated ions of selected deuterated analytes in relation to the theoretical mass over a period of 55h.

In general, LOQs varied between 4 and 68 ng L^{-1} in influent sewage water and between 2 and 35 ng L^{-1} in effluent sewage water (**Table 2**). As previously described, theoretical LOQs can be calculated using the lowest calibration standard divided by the matrix suppression. For MDA, morphine and THC high suppression resulted in too high theoretical LOQs (influents: 439, 486 and 29,221 ng L^{-1}, respectively; effluents THC: 5747 ng L^{-1}). However in practice, concentrations lower than these theoretical LOQs could be satisfactorily quantified. For these compounds, the concentrations in spiked samples that were used for method validation were used as LOQs (360 ng L^{-1} for all three analytes in influent and 180 ng L^{-1} for THC in effluent). Analyte- or analogue-ILIS were used for all drugs of abuse as shown in **Table 1**, with the exception of heroine,

methadone, ketamine, methylphenidate and mCCP, for which no appropriate ILIS was found.

Recoveries, calculated from relative responses analyte/ILIS, were tested for influent and effluent samples spiked at 360 ng L^{-1} and 180 ng L^{-1}, respectively, which led to a concentration of 72 µg L^{-1} in the final extract (**Table 2**). Relative recoveries for most of the drugs in each matrix were between 70 and 120%, with the exception of mCCP (influent, 52% and effluent, 62%), methylphenidate (influent, 45% and effluent, 65%) and ketamine (influent, 48%). Precision was <30% for heroin, methadone, codeine, ketamine, methylphenidate, desalkyl-flurazepam, mCCP and fentanyl, for the remaining compounds it was ≤15% (**Table 2**). Best recoveries and precision were observed for those compounds that could be corrected by the use of ILIS, illustrating the importance of adequate matrix correction. For the 5 compounds of which no ILIS were available recovery data were obtained without ILIS correction, and were poorer as expected, ranging from 45% (methylphenidate in influent) to 81% (ketamine in effluent). In the analysis of sewage water samples (see next section), results for these 5 compounds were corrected using quality control (QC) recoveries, included in every sequence of analysis. The fortified sewage waters (QC) selected for correction were taken from the same STP during the same sampling period in order to minimize matrix composition differences due to temporal variations or different locations, obtaining a sample set with a more uniform matrix [23]. Although results always need to be critically evaluated, this approach was considered satisfactory in absence of appropriate ILIS, as the treatment procedure for samples and QCs (fortified sewage water) is the same and matrix effects are expected to be comparable for samples taken within the same period of sampling and within the same STP.

3.4. Application to sewage water samples

The strong potential of Orbitrap for identification purposes comes from its high resolution, as recently illustrated for different organic pollutants [6, 8, 24]. However, Orbitrap has been much less used for quantification purposes. The main objective of the present work was to demonstrate that a confident quantification can also be

made by Orbitrap for drugs of abuse in sewage water in addition to its, already expected, excellent capabilities for identification/elucidation purposes.

The methodology developed was applied to sewage water samples collected from the Netherlands. The samples formed part of a pilot study on drugs of abuse in which two other laboratories also participated analyzing the same samples but applying different methods, all based on the use of LC–MS/MS with triple quadrupole. The analytical methodologies were in-house validated using ILIS for matrix effects and sample handling errors correction. The most frequently detected drugs were MDMA, benzoylecgonine, morphine, codeine, oxazepam and temazepam, which were present in at least 75% of influent and effluent sewage water samples. The highest drug levels were found in influent sewage water and corresponded to oxazepam (average concentration 1167 ng L^{-1}) and benzoylecgonine (average concentration 1703 ng L^{-1}), the main metabolite of cocaine, highlighting the widespread consumption of benzodiazepines and cocaine. Data from this study and more detailed information can be found elsewhere [25, 26].

The fact that samples were analyzed by different techniques allowed us to perform an additional validation step of the analytical method applied, a relevant aspect taking into account that LC–MS/MS with QqQ is the most widely applied technique for the determination of drugs of abuse in sewage water. This could be done for six target drugs that were included by all participants and were detected in several of the samples analyzed. **Table 3** shows the concentrations found in four of these samples, which have been taken as illustrative examples because they were positive for several analytes included in this work. In this table, data reported for two laboratories using LC–MS/MS QqQ are compared with our data using Orbitrap.

Table 3: Comparison of concentrations of drugs of abuse detected in influent and effluent sewage waters analyzed by LC-MS/MS with triple quadrupole (two different laboratories) and by the Orbitrap method presented in this work. Each number is the result of a single measurement of the pertaining sample using a specific MS detection technique

Compound	Influent sewage water (ng L^{-1})						Effluent sewage water (ng L^{-1})					
	Sample 1			Sample 2			Sample 3			Sample 4		
	QqQ 1[a]	QqQ 2[b]	Orbitrap	QqQ 1	QqQ 2	Orbitrap	QqQ 1	QqQ 2	Orbitrap	QqQ 1	QqQ 2	Orbitrap
Amphetamine	95	117	123	282	249	245	-	-	-	-	-	-
MDMA	21	96	144	-	56	86	84	88	137	50	54	76
Cocaine	439	296	d[c]	179	114	d[c]	2	-	-	14	-	8
Benzoylecgonine	1178	1136	1637	528	645	615	26	19	45	85	77	99
THC-COOH	378	n/a[d]	d[c]	-	n/a	-	-	n/a	-	-	n/a	-
Ketamine	n/a	-[e]	-	n/a	-	-	n/a	-	2	n/a	16	6

[a] Pre-treatment: centrifugation; Pre-concentration by SPE (Oasis MCX, 150 mg); pre-concentration factor: influent 10x, effluent, 50x; [29]

[b] Pre-treatment: none; Pre-concentration by SPE (Oasis HLB, 200 mg); pre-concentration factor: influent 50x, effluent 250x; [25]

[c] d: detected

[d] n/a: no data available

[e] - : not detected

Data for amphetamine, MDMA and benzoylecgonine were, in general, in good agreement, with the exception of MDMA in influent sample 1 for QqQ 1, where the concentrations reported were notably lower than by QqQ 2 and Orbitrap. The overall (inbetween laboratories) deviation for these three analytes was ≤30%, with the above mentioned exception of MDMA in influent sample 1 and benzoylecgonine found at trace level in effluent sample 3. Few data were available for THC-COOH and ketamine. However, it can be noticed that Orbitrap was able to detect THC-COOH in the only sample that was positive by this compound (influent sample 1, QqQ 1), although it could not be quantified despite the concentration reported by QqQ was higher than the LOQ estimated for Orbitrap in influent. In relation to ketamine, in the effluent sample 4 where this compound was quantified by QqQ 2 (16 ng L^{-1}), the concentration reported by Orbitrap was about the same order (6 ng L^{-1}). Data for cocaine were less consistent, although it could be detected by Orbitrap in the two influent samples, and the level reported in the effluent 4 (8 ng L^{-1}) was about the same order as that obtained by QqQ 1 (14 ng L^{-1}). More data are required in future monitoring to have a more realistic overview of the Orbitrap applicability to THC-COOH and cocaine analysis. In the time of writing this report, an intercomparison study between 13 laboratories has been made for several illicit drugs (including those mentioned above), which will shed more light on this issue. These inter-laboratory comparison data will be the subject of a specific publication in the very near future. In addition to the six common compounds monitored by all participants, other target drugs could be quantified by Orbitrap in these four samples. In both influents, codeine, oxazepam and temazepam were also found. In the effluents, codeine, oxazepam, temazepam, nordazepam, diazepam (sample 4) and methylphenidate (sample 3) were also detected.

As an illustrative example, **Fig. 4** shows $[M+H]^+$ extracted-ion chromatograms (exact mass ± 5 ppm) and MS/MS spectra for several drugs of abuse detected in influent and effluent samples.

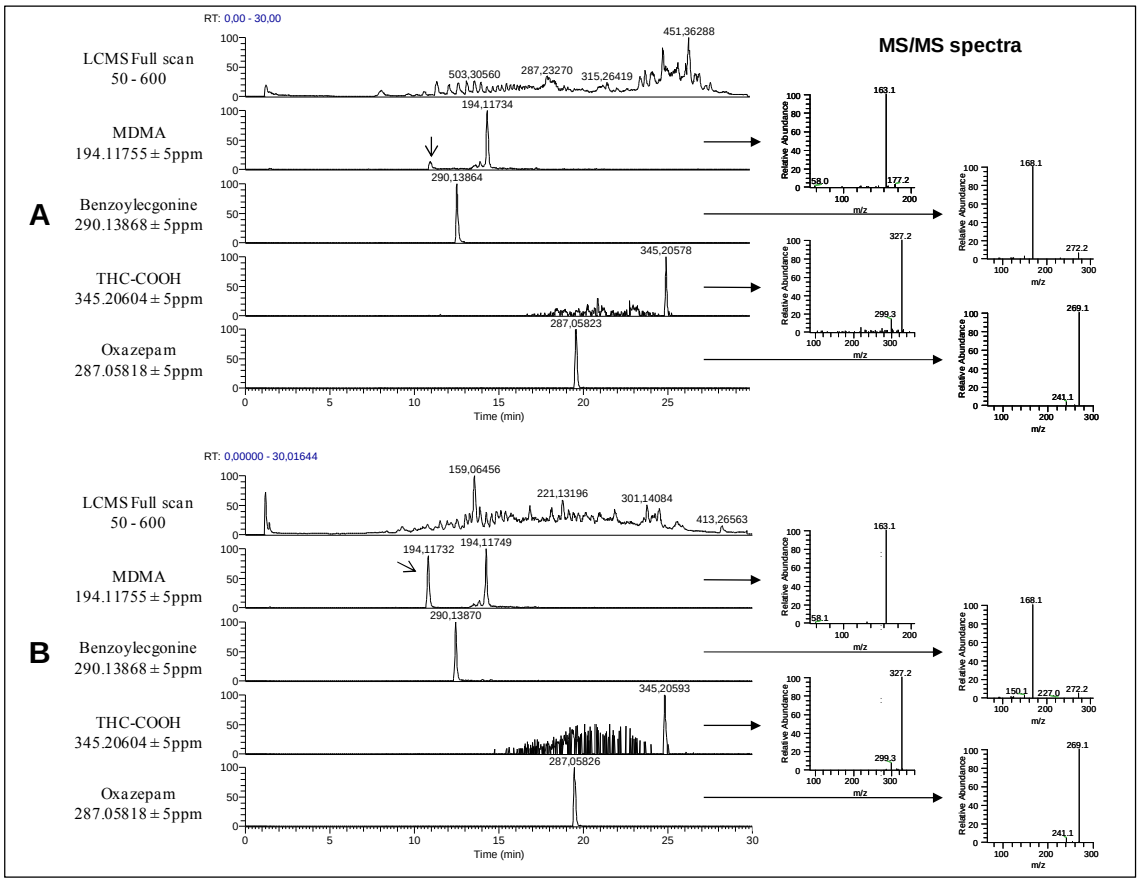


Figure 4. LC-MS (ESI + mode) extracted-ion chromatograms (left) and MS/MS spectra (right) of drugs of abuse detected in influent (A) and effluent (B) sewage water from the sewage treatment plant of Amsterdam. Concentrations found in these samples were the following (influent and effluent, respectively); MDMA: 136 and 190 ng L^{-1}; benzoylecgonine: 3701 and 155 ng L^{-1}; THC-COOH: 431 and 22 ng L^{-1}; Oxazepam: 430 and 422 ng L^{-1}. Arrows indicate chromatographic peak of MDMA.

An advantage of HRMS, derived from the full-acquisition accurate mass data, is the possibility to perform retrospective analyses [27]. Although it was not the objective of our work, we briefly explored this feature in order to tentatively confirm the presence of ketamine in several samples. Occasionally, ketamine was suspected to be present, based on the finding of the accurate mass of the $[M+H]^+$ ion at the expected retention

time. However, due to the high matrix complexity, the product ions, normally used for confirmation of the identity of ketamine, could not be detected in the samples These samples were considered on a case-by-case basis, and were further investigated retrospectively thanks to the useful information acquired by LTQ-FT Orbitrap searching for the ketamine metabolites norketamine and dehydronorketamine. Although it is not clear if the latter is a true metabolite or an artefact, both compounds have been largely found in urine, even at concentrations higher than ketamine [28]. Hence one would expect to find these compounds in sewage water, assuming no further degradation in the sewer. **Fig. 5** shows the retrospective search of norketamine and dehydronorketamine using their exact masses with a maximum error of 5 ppm. The presence of chromatographic peaks in the extracted ion chromatograms at the exact masses of the two metabolites is an indication of their presence in the sample analyzed. One may conclude from this observation that ketamine is likely to be present in the sample. Obviously, more research would be required to unequivocally confirm the presence of these metabolites, e.g., injecting reference standards, but these findings are illustrative of the potential of the Orbitrap analyzer.

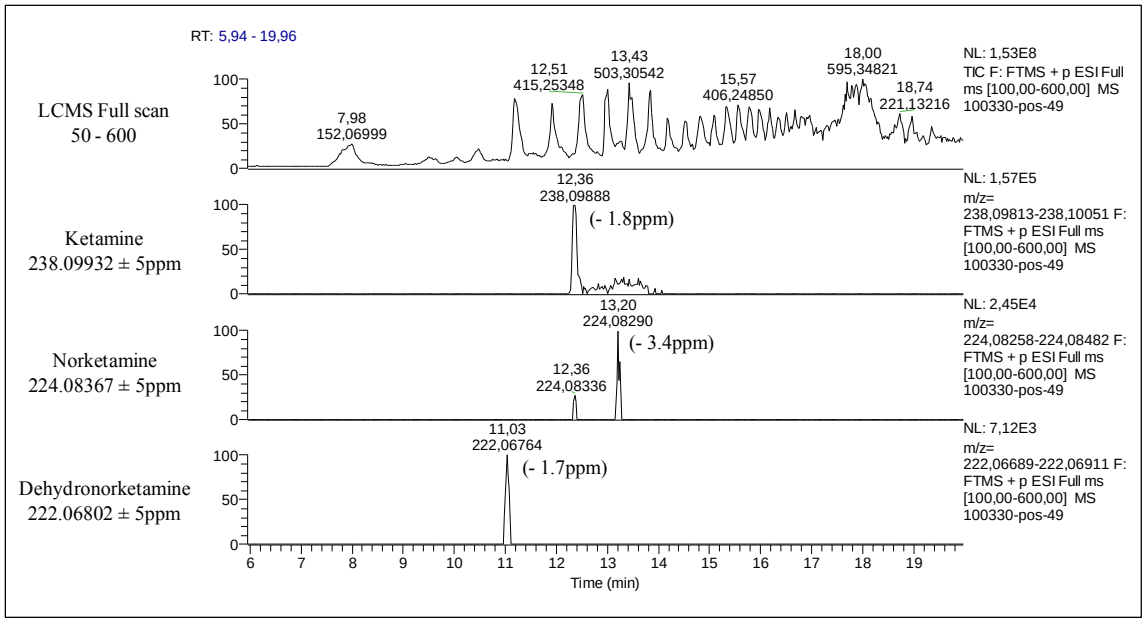


Figure 5. LC-MS (ESI+ mode) extracted-ion chromatograms of ketamine, norketamine and dehydronorketamine in an influent sewage water sample from Eindhoven (retrospective search).

4. Conclusions

Analytical methodology based on full-spectrum accurate-mass and MS/MS acquisition provided by LC-LTQ FT Orbitrap MS has been developed for the simultaneous quantification and confirmation of 24 target drugs of abuse at ng L^{-1} levels in sewage water. Although Orbitrap is recognized as an excellent analyzer for qualitative purposes, its suitability to perform quantitative analysis has not been much explored. In this work, Orbitrap has been applied for the first time to the quantitative analysis of drugs of abuse in sewage water. Our data showed that this analyzer can be used for the reliable quantification with almost the same sensitivity as the most commonly used methodologies based on LC–MS/MS with triple quadrupoles. The quantitative applicability has been demonstrated by method validation and the analysis of quality control samples included in each sample sequence, and also via a comparison with data reported by triple quadrupole analysis. In addition, MS data provided by Orbitrap have allowed retrospective analysis leading to an indication of the presence of two ketamine metabolites. In conclusion, this unique feature of high-resolution accurate-mass spectrometry demonstrates that ketamine is likely to be present in several samples.

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References

[1] E. Zuccato, S. Castiglioni, R. Bagnati, C. Chiabrando, P. Grassi, R. Fanelli, Water Res. 42 (2008) 961-368.

[2] D.R. Baker, B. Kasprzyk-Hordern, J. Chromatogr. A 1218 (2011) 8036-8059.

[3] M.R. Boleda, M. Huerta-Fontela, F. Ventura, M.T. Galceran, Chemosphere 84 (2011) 1601–1607.

[4] A.L.N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M. Lopez de Alda, H. Neels, E. Zuccato, D. Barcelo, A. Covaci, Sci. Total Environ. 409 (2011) 3564-3577.

[5] M. Kellmann, H. Muenster, P. Zomer, H. Mol, J. Am. Soc. Mass Spectrom. 20 (2009) 1464-1476.

[6] M. Krauss, H. Singer, J. Hollender, Anal. Bionanal. Chem. 397 (2010) 943-951.

[7] F. Hernández, J.V. Sancho, M. Ibáñez, E. Abad, T. Portolés, L. Mattiolo, Anal. Bioanal. Chem. 403 (2012) 1251-1264.

[8] A.C. Hogenboom, J.A. van Leerdam, P. de Voogt, J. Chromatogr. A 1216 (2009) 510-519.

[9] F. Hernández, L. Bijlsma, J.V. Sancho, R. Díaz, M. Ibáñez, Anal. Chim. Acta 684 (2011) 96-106.

[10] J. Nurmi, J. Pellinen, J. Chromatogr. A 1218 (2011) 6712-6719.

[11] I. González-Mariño, J.B. Quintana, I. Rodríguez, M. González-Díez, R. Cela, Anal. Chem. 84 (2012) 1708-1717.

[12] J.A. van Leerdam, A.C. Hogenboom, M.M.E. van der Kooi, P. de Voogt, Int. J. Mass Spectrom. 282 (2009) 99-107.

[13] A. Kaufmann, P. Butcher, K. Maden, S. Walker, M. Widmer, Rapid Commun. Mass Spectrom. 25 (2011) 979-992.

[14] S. Vogliardi, D. Favretto, M. Tucci, G. Stocchero, S.D. Ferrara, Anal. Bioanal. Chem. 400 (2011) 51-67.

[15] R. Pinhancos, S. Maass, D.M. Ramanathan, J. Mass Spectrom. 46 (2011) 1175-1181.

[16] J.M. Marín, E. Gracia-Lor, J.V. Sancho, F.J. López, F. Hernández, J. Chromatogr. A 1216 (2009) 596-612.

[17] O.J. Pozo, J.V. Sancho, M. Ibáñez, F. Hernández, W.M.A. Niessen, Trends Anal. Chem. 25 (2006) 1030-1042.

[18] Commission Decision 2002/657/EC of 12 August implementing Council Directive 96/23/EC concerning performance of analytical methods and the interpretation of results. Off. J. Eur. Commun. L221/8 (2002).

[19] A. Kaufmann, M. Widmer, K. Maden, Rapid Commun. Mass Spectrom. 24 (2010) 2162-2170.

[20] P. de Voogt, E. Emke, R. Helmus, P. Panteliadis, J.A. van Leerdam, in: S. Castiglioni, E. Zuccato, R. Fanelli (Eds), Mass Spectrometric Analysis of Illicit Drugs in the Environment, Wiley, New York, 2011, pp. 85-114.

[21] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019-3030.

[22] S. Castiglioni, E. Zuccato, E. Crisci, C. Chiabrando, R. Fanelli, R. Bagnati, Anal. Chem. 78 (2006) 8421–8429.

[23] M. Stüber, T. Reemtsma, Anal. Bioanal. Chem. 378 (2004) 910-916.

[24] R.J.B. Peters, A.A.M. Stolker, J.G.J. Mol, A. Lommen, E. Lyris, Y. Angelis, A. Vonaparti, M. Stamou, C. Georgakopoulos, M.W.F. Nielen, Trends Anal. Chem. 29 (2010) 1250-1268.

[25] N.G.F.M. van der Aa, E. Dijkman, L. Bijlsma, E. Emke, B.M. van de Ven, A.L.N. van Nuijs, P. de Voogt, RIVM report 703719064, Bilthoven, (2010) pp. 1-90. Available from URL: www.rivm.nl (accessed September 2012)

[26] N.G.F.M. van der Aa, L. Bijlsma, E. Emke, E. Dijkman, A.L.N. van Nuijs, B.M. van de Ven, F. Hernández, J.F.M. Versteegh, P. de Voogt, Water Res. 47 (2013) 1848-1857.

[27] F. Hernández, M. Ibáñez, E. Gracia-Lor, J.V. Sancho, J. Sep. Sci. 34 (2011) 3517-3526.

[28] K-C. Wang, T-S. Shih, S-G. Cheng, Forensic. Sci. Int. 147 (2005) 81-88.

[29] L. Bijlsma, J.V. Sancho, E. Pitarch, M. Ibáñez, F. Hernández, J. Chromatogr. A 1216 (2009) 3078-3089.

3.3 Discussion of the results

High resolving power and high mass accuracy

The high resolving power of TOF MS and FT Orbitrap MS increases selectivity against the matrix background and permits to measure accurately the mass of a compound and the assignment of a reliable elemental composition, with the final identification of the compound detected.

In this chapter, both QTOF MS and LTQ Orbitrap MS were used. These instruments were able to measure mass accuracy better than 5 ppm. The TOF MS instrument permitted a mass resolution over 20,000 FWHM, whereas the Orbitrap MS allowed obtaining spectra at a mass resolution of up to 100.000 FWHM. Accordingly, this means that the relative limited resolution of TOF MS, compared to FT Orbitrap MS, may lead to higher mass errors in complex matrices, because of unresolved background matrix interferences. Yet, a drawback of applying an increased resolution in TOF MS instruments is the decrease in sensitivity, which is not beneficial for a wide-screening of environmental samples where concentrations of some compounds of interest can be expected to be low. Regarding Orbitrap MS instruments, an increased resolution results in an increase in the total cycle time, e.g. at a resolution of 100,000 FWHM the scan time is 1s. A reduced scan speed makes the instrument less suitable to detect the sharp peaks (< 5 s) that are generated, for example, under UHPLC conditions. The relative slow data acquisition rate of the Orbitrap makes it often incompatible with UHPLC, at least if the aim is to analyze several types of data within a single LC-MS run. New generation Orbitraps are faster and combinations with UHPLC might be feasible. Unfortunately, the recently published papers do not fully take profit of these new developments [Pinhancos *et al.* 2011; Wille *et al.* 2011; Rodayan *et al.* 2014]. As for the first Orbitrap instruments, used in *scientific article 5*, a compromise between mass resolving power and chromatographic peak shape need to be reached, since the total cycle time depends upon the resolution.

In *scientific article 5*, where an HPLC-LTQ Orbitrap MS was employed for qualitative and quantitative analysis of 24 IDs, a satisfactory compromise was obtained when mass

resolution was set at 30,000 FWHM. The total cycle time of about 0.55s (including the simultaneous MS/MS acquisition in the ion trap) allowed at least ten data points across each chromatographic peak. Conversely, QTOF MS as applied in *scientific article 3 and 4*, is compatible with UHPLC due to its high speed acquisition capabilities. UHPLC provides increased resolution and shorter run time, important parameters in terms of sample throughput and sensitivity improvement. Moreover, the gain in separation efficiency minimizes component co-elution, which is especially important for QTOF MS when running in MS^E acquisition mode, as in this mode co-eluting components can enter the collision cell simultaneously; complicating the interpretation of the combined (LE and HE) mass spectra. UHPLC results in the minimization of matrix interferences, and consequently renders in high mass spectra purity, improving screening processes.

In addition to the importance of minimizing matrix interferences, mass accuracy is essential for the correct identification of a compound. Therefore, control of mass accuracy is a key factor in assuring the reliability of results.

The use of reference sprayers, such as lock-spray, improves robustness in mass accuracy measurement along time. TOF systems switch intermittently from LC flow to a reference solution which is continuously sprayed. This allows continuous automated calibration of the mass axis. In contrast to TOF systems, continuous calibration is not necessary for the Orbitrap analyzer as mass accuracy remains within a deviation of 5 ppm over a period of at least 55h (***figure 3, scientific article 5***). Yet, mass calibration has to be performed with every batch run just prior to starting the batch.

Target analysis, quantification

The identification and quantification of IDs in the aquatic environment at low concentration requires both high sensitivity and selectivity against complex matrix backgrounds [Krauss *et al.* 2010]. Low resolution LC-MS/MS instruments have demonstrated excellent performances for quantitative analysis. However, there are some limitations associated to these instruments, such as the limited number of compounds that can be monitored in one run. Furthermore, for some analytes with low *m/z* values for their precursor ions (e.g. amphetamine class drugs), it is problematic to

obtain two intense transitions, which are required for proper identification in the SRM mode [Castiglioni *et al.* 2008; Krauss *et al.* 2010; Gonzalez-Mariño *et al.* 2012]. Conversely in some cases a "too rich" MS/MS fragmentation pattern might cause significant loss of sensitivity [Castiglioni *et al.* 2008; Gonzalez-Mariño *et al.* 2012].

The increasing interest of using LC-HRMS(/MS) relies on its capability to perform both target as well as non-targeted analysis, based on full-spectrum accurate-mass acquisition at good sensitivity [Hernández *et al.* 2012]. It opens the possibility to identify compounds with improved reliability and robustness. HRMS precursor and product ions (R > 20,000 FWHM) earn 2 and 2.5 identification points, respectively, instead of 1 and 1.5 IPs for low resolution MS. Thus, one single high resolution MS/MS transition is enough to fulfill the identification point's guideline [2002/657/EC], where 3 IPs (4 IPs for banned compounds) are needed for confirmation of the compounds detected.

Although the strong potential of HRMS for identification purposes is widely recognized, one of the main criticisms is its relative low sensitivity and low dynamic range compared with QqQ operating in SRM mode. Yet, these limitations particularly concern the first-generation TOF MS instruments. The linearity range of these TOF measurements was limited because of the way that ions were detected. Early TOF MS systems utilized time to digital converters (TDC) based circuitry to perform high speed digitization of signals generated from the TOF multi-channel plate detectors. This TDC detection proved to be effective, but suffered from dynamic range limitations. More recently, TOF MS instruments utilize analog to digital converters (ADC) based digitization, greatly improving their linear dynamic range [Gilbert *et al.* 2009; Kaufmann *et al.* 2009]. Therefore improvements in detection, but also in resolving power (> 40,000 FWHM) of the latest TOF instruments provide better quantitative and qualitative capabilities [Gonzalez-Mariño *et al.* 2012]. As for Orbitrap instruments, the LC-LTQ Orbitrap MS used in *scientific article 5* showed a good dynamic range and sensitivity close to that of many QqQ instruments, allowing both quantification and confirmation in a single analytical run. (Data shown in ***table 3, scientific article 5***; and Castiglioni *et al.* 2013 – *scientific article b*; van der Aa *et al.* 2013 – *scientific article 10*). Accordingly, the Orbitrap MS has been applied to wastewater samples collected in the Netherlands. The

most frequently detected drugs were amphetamine, MDMA, cocaine, BE, THC-COOH and the pharmaceutical codeine, oxazempam and temazepam, with mean concentrations up to 2306 ng/L for BE in influent wastewaters. Data and more detailed information can be found in Chapter 5 (van der Aa *et al.* 2013 – *scientific article 10;* Bijlsma *et al.* 2012 – *scientific article* 9).

Quantification by UHPLC-QTOF MS was not performed in *scientific article 3 and 4*. Firstly, the objective was to perform a wide-scope screening of 76 IDs and standard solutions were not available for all compounds to perform quantification. Secondly, for the 11 compounds for which reference standards were available, quantification by QTOF MS (QTOF Premier, Waters Micromass) did not seem to be the most attractive feature compared to QqQ (TQD, Waters) used in *scientific article 1;* therefore, the work was focused on qualitative aspects only.

Post-target analysis

Post-target analysis is an interesting approach that offers the possibility to screen for a large number of IDs, taking profit of the high quality data obtained from HRMS, without being dependent on the pre-selection of analytes or immediate need of reference standards. The latter is very beneficial, since most of the ID standards are expensive, quite limited in the amount commercially available, and it is often time-consuming to purchase them. Hence, the possibility of acquiring reference standards only after tentative identification for the final confirmation is very attractive. In addition, the presence of compounds can be investigated from accurate mass data acquired at any time without the need of additional analysis of the samples. This allows widening screening of compounds by only reprocessing raw data in a retrospective way. This may become very useful for the detection of new drugs, such as new psychoactive substances (NPS), which continuously appear on the market [EMCDDA 2012].

The applicability of the post-target approach by LC-QTOF MS has been demonstrated in *scientific articles 3 and 4*. The full-spectrum acquisition data, generated under MS^E mode, were inspected based on the use of a home-made database. Initially, the name and elemental composition of the compounds were the only information

included in the customized database (***table 1, scientific article 3***). However, this database was continuously modified and improved. Information about fragmentation reported in the literature was also included, as well as empirical data (e.g. retention time and in-source fragment ions) obtained from IDs of which reference standards were available. Currently the database built at the IUPA contains around 2000 compounds, including pesticides, mycotoxins, veterinary drugs, pharmaceuticals, personal-care products, IDs, NPS, etc (**Figure 3.2**). The database is used in combination with the application manager ChromaLynx XS (within MassLynx). Powerful software, such as ChromaLynx, facilitates the data processing and might even perceive (low abundant) compounds overlooked by visual inspection. It offers the possibility of applying a "post-target" processing method based on monitoring theoretical exact masses of the selected analytes (included in the database) using narrow mass extraction windows (nw), commonly 10-20 mDa, and permits a rapid and simple reviewing by classifying candidates, as a function of the mass error. In addition, this software allows the simultaneous visualization of the complete mass spectra of positive findings at LE and HE functions. In this post-target screening, the presence of the protonated molecule measured at its accurate mass is searched in the samples. For this purpose, nw-XICs at the *m/z* of all compounds included in the database are automatically performed. After that, fragment ions, typically in the HE function, and characteristic isotopic ions are further evaluated [Ibáñez *et al.* 2014].

The described methodology for rapid screening of IDs has been applied to 20 wastewater samples (ten influent and ten effluent samples). Ten compounds were detected and identified, of which, cocaine, BE and codeine were the most frequently detected (***table 2, scientific article 3***). Reference standards were available for cocaine, BE, norBE, MDMA, and methamphetamine and their presence could, therefore, easily be confirmed. For the other compounds (codeine, cotinine, ketamine, oxazepam and temazepam), of which reference standards were not initially available, the approach has resulted in tentative identifications as demonstrated in ***figure 3 of scientific article 3*** and ***figure 4.3 of scientific article 4***. However, despite the amount of evidence presented, the identities of positive findings could only be confirmed unequivocally

after acquiring reference standards and performing additional analysis. Yet, only reference standards were necessary for the positively identified IDs.

	A	B	C	D
1	**Compound name**	**Empiricalformula**	**t_R**	**$[M+H]^+$**
183	**MDEA**	C12H17NO2	3.48	208.1337
184	MDEA F1	C9H8O	3.48	133.0653
185	MDEA F2*	C8H6O2	3.48	135.0446
186	MDEA F3	C8H8	3.48	105.0704
187	MDEA F4	C6H4	3.48	77.0391
188	**MDMA**	C11H15NO2	3.02	194.1181
189	MDMA F1*	C10H10O2	3.02	163.0759
190	MDMA F2	C8H8	3.02	105.0704
191	MDMA F3	C8H6O2	3.02	135.0446
192	MDMA F4	C9H8O	3.02	133.0653
193	MDMA F5	C6H6	3.02	79.0548
194	**Mephedrone**	C11H15NO		178.1232
195	**Methadone**	C21H27NO		310.2178
196	**Methamphetamine (METH)**	C10H15N	3.05	150.1282
197	Methamphetamine F1	C9H10	3.05	119.0861
198	Methamphetamine F2	C7H6	3.05	91.0548
199	Methamphetamine F3	C5H4	3.05	65.0391
200	**Methcathinone**	C10H13NO	2.26	164.1075
201	Methcathinone F1*	C10H11N	2.26	146.0969
202	Methcathinone F2	C9H8N·	2.26	131.0735
203	Methcathinone F3	C8H8	2.26	105.0704
204	Methcathinone F4	C8H6	2.26	103.0547
205	**Methedrone**	C11H15NO2		194.1181

Veterinary drugs ESI+ | Veterinary drugs ESI- | Pharmaceutical metabolites | Viagras | **Illicit drugs** | Hormones | Surfactants | UV filters

Figure 3.2: Screen-shot of the home-made database, currently available at IUPA.

Post-target analysis using LTQ Orbitrap MS is also feasible, taking advantage of the accurate mass measurements in combination with the trapping capacity and MS^n scan function of the linear ion trap. As previously commented, the MS can operate in a data-dependent-acquisition (DDA) mode in which both MS and MS^n spectra can be acquired without the need to specify precursor masses. In this mode, the acquisition software probed the MS spectra in real-time on a scan-by-scan basis to select the most intense precursor ions for MS^n analysis. The instrument is initially set to operate in full-scan ('survey') mode with accurate mass measurements, until an ion/ions exceed/s a preset threshold. Moreover, an inclusion list of target ions of interest can be defined and at which point the instruments switches into the product-ion scan mode (MS^n). A limiting factor is the relative long cycle time of LTQ-Orbitrap MS. This is more noticeable when dealing with complex samples, where the amount of consecutive chromatographic

peaks exceeding the threshold can be too high for the system in order to acquire both MS and MS^n spectra in a chromatographic time fashion. Consequently, valuable information for identification purposes will not be available. In addition, the MS/MS spectra obtained in the linear ion trap usually show only one or two nominal-mass product ions, while mass spectra obtained in the collision cell of the QTOF at the HE mode are richer in the presence of accurate-mass fragment ions, giving more information that facilitates the subsequent identification process.

Although it was not the objective within *scientific article 5*, post-target analysis has also been performed using LTQ Orbitrap MS. In order to gain more evidence on the presence of ketamine in some of the samples analyzed, a retrospective search of its metabolites, norketamine and dehydronorketamine, had been performed using their exact masses (± 5 ppm). Data is shown in ***figure 5 of scientific article 5***. The presence of chromatographic peaks might be considered as an indication of their presence as well as the presence of ketamine in the samples analyzed. Obviously, more research is required for unambiguous identification.

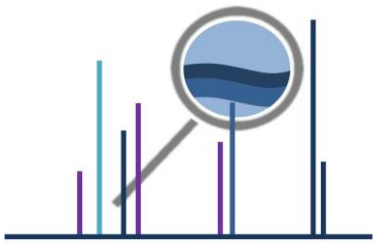
Non-target analysis

In contrast to post-target screening, non-target (unknown) screening in a strict sense starts without any *a priori* information on the compounds to be investigated. Identifying "unknown compounds" is challenging and beyond the strategy followed in this Thesis. Nevertheless, the potential of HRMS for non-target screening will briefly be discussed.

LC-HRMS is an accepted technology for generating meaningful structure suggestions of unknowns. To reach a tentative identification, non-target screening normally involves a workflow consisting of several evaluation steps. When screening a sample for unknown compounds, it becomes difficult to pick out individual ions, especially when the matrix is complicated or when the concentration of the compound is low. Under these circumstances, it is necessary to use powerful software with chromatographic peak deconvolution capabilities to identify the presence of multiple components and to produce pure spectra for each individual component. Once a pure spectrum has been generated, an elemental composition (empirical formula) has to be assigned to

the accurate mass of interest. Then, a library search for plausible structures of the determined elemental composition has to be performed and the chemical structures found in libraries (e.g. home-made database, NIST library and internet sites such as chemfinder (www.chemfinder.com) and Chemspider (www.chemspider.com)) need to be evaluated based on the fragmentation patterns. Obviously, this process requires time and expertise of the researcher. The possibilities of success are rather limited at the moment, as several potential chemical estructures may be suggested in most cases for a given candidate.

Both QTOF MS and LTQ Orbitrap MS can be applied for this challenging task. However, the high resolving power (up to 100.000 FWHM) of Orbitrap in combination with the possibility to perform MS^n experiments make this hybrid instrument probably best suited for the identification of unknowns. Using LTQ-Orbitrap MS, full-scan accurate mass measurements allow the proposal of an elemental composition, which can subsequently be used to search for possible structures. Furthermore, MS^n data, generated in the linear ion trap (nominal mass of product ions) within the same analysis, are used to obtain information of fragment ions. In addition, the accurate masses of these product ions can be obtained in a second analysis. The masses of these fragment ions can then be linked with precursor compound masses and ordered in a so-called "fragmentation tree". This workflow allowed Hogenboom *et al.* (2009) to tentatively identify an unknown (polar) organic compound in groundwater as metolachlor OA.



CHAPTER 4

Fragmentation pathways and transformation of illicit drugs

4.1 Introduction

A detailed knowledge of the fragmentation pathways of different classes of IDs is of great interest from an analytical point of view. For example, this information is valuable for the selection of specific fragment ions when developing MS/MS based analytical methods.

The choice of specific product ions is highly relevant for the selection of appropriate SRM transitions in quantitative analyses using QqQ. Commonly, the most abundant transitions are selected in order to increase method sensitivity; however, less attention is usually paid to mass spectrometric issues as, for example, the losses involved and the specificity of the transition. False positives or an overestimation of the concentration may be reported if the selected transition is not specific enough; therefore transitions such as those involving the loss of H_2O or CO_2 should be avoided. Nonspecific transitions may also lead to reporting false negatives when matrix interference shares one of the transitions with the analyte, affecting the compliance of the ion ratio [Pozo *et al.* 2006]. The probability of reporting false positives or false negative increases when dealing with analytes at sub-ppb levels in complex matrices, e.g. the determination of IDs in urban wastewater. Thus, for the selection of product ions in SRM acquisitions, not only their

abundance but also their specificity should be taken into account as it is of great relevance for trace-level analysis.

LRMS analyzers, such as QqQ and IT, have been applied for the identification and elucidation of fragment ions and demonstrated that nominal-mass measurements can be useful for this purpose [Wang and Bartlett, 1998; Jeanville *et al.* 2003; Maralikova and Weinmann, 2004; Smyth, 2003; Wang *et al.* 2005]. However, the high resolution and mass accuracy provided by HRMS allow the investigation of the fragmentation pathways and elucidation of structures with higher confidence. The power of accurate mass relies on the determination of the elemental composition of a compound or its fragment ions. Two aspects of the measurement of accurate mass lead to a correct empirical/molecular formula. The mass accuracy and the isotopic distribution pattern (accuracy and intensity). Used together these two types of information are valuable to obtain the correct formula [adapted from Thurman and Ferrer, 2009]. The molecular formula derived from the measured accurate *m*/z, together with the use of basic criteria, such as the nitrogen rule (**Table 4.1**), double bond equivalent (DBE), and the concept that fragmentation should involve logic neutral losses and no major rearrangements in the structure of the drug, allow the proposal of plausible structures of fragment ions.

Table 4.1. Nitrogen rule

	Zero or even Nitrogen atoms	Odd Ntrogen atoms
Odd Electron ion ($OE^{+\cdot}$)	**Even *m*/z**	**Odd *m*/z**
Even Electron ion (EE^{+})	**Odd *m*/z**	**Even *m*/z**

Note[a]: the rule applies when a neutral organic molecule has only carbon, hydrogen, oxygen, nitrogen, sulfur, phosphorus, or any halogen

Note[b]: LC-MS data are mainly dominated by even electron ions

The knowledge of fragment ions of IDs is not only essential for the selection of appropriate (selective) SRM transitions, but also when searching for drug-related compounds. Once entering in the sewer system IDs and their metabolites are subjected to degradation/transformation processes that can lead to their complete

mineralization to inorganic end products, such as CO_2, H_2O, NH_4^+. However, when the mineralization process is not carried to completion or when it occurs slowly in the environment, different intermediate compounds can be formed. Assuming that most of these compounds share similar fragmentation pathway with the parent drug, searching for precursor ions of common fragments may lead to the discovery of unknown TPs. So, knowledge of structures of fragment ions and basic fragmentation rules are also helpful for achieving confident TP structure proposals. In many cases, these TPs have unknown physico-chemical properties, and might exhibit high toxicity, even more than the parent compound, producing undesirable negative effects on humans and wildlife [Farré *et al.* 2008; Fatta-Kassinos *et al.* 2011]. Hence, the discovery of TPs is important to understand the overall contribution of any organic contaminant, and particularly of IDs, in the environment.

LC–QTOF MS, when using a soft ionization source such as ESI, is a powerful technique for identification and confirmation purposes. Besides the high mass accuracy provided by the TOF analyzer, it offers the possibility to perform data acquisition in conventional MS/MSor in MS^E (*i.e.* pseudo MS/MS) mode. In MS/MS mode, specific precursor ions are isolated for further analysis. The product ion (accurate) mass spectra obtained are generally cleaner, especially when applied to complex matrices. Conversely in MS^E mode, no pre-selection is needed and accurate mass data of the (de)protonated molecules, their isotopic pattern and fragment ions are acquired nearly simultaneously. The latter approach, which is explained in more detail in chapter 3 of this thesis, is very useful for the identification and elucidation of TPs, where precursor ions are unknown to the analyst and therefore cannot be pre-selected for MS/MS acquisitions.

When searching for unknown TPs a target approach is obviously not feasible. Conversely a true non-target screening, without any *a priori* information on the compounds to be detected, is an analytical challenge, the process is time consuming and needs expertise, and the possibilities of success are rather limited [Ibañez *et al.* 2005; Krauss *et al.* 2010; Hug *et al.* 2014]. Therefore, different alternative strategies based on accurate mass measurements have been suggested for the identification of drug-related compounds and will be explained in continuation.

Strategy 1 *– prediction based on common fragmentation pathways and/or mass defect filtering*

An intermediate situation between targeted and non-targeted screening is directed towards the discovery of compounds related to known IDs on the basis of ***common fragmentation pathways***. The proposed strategy is based on: (i) the assumption that TPs maintain a similar structure than the parent compound and therefore originate common product ions, and (ii) the search for precursor ions of the expected common fragments. Narrow-mass window XICs (e.g. ± 0.01Da) for specific fragment ions can be obtained from the HE and/or LE functions of the MS^E data. When these chromatograms show additional chromatographic peaks (at retention times different to that of the parent drug), the presence of parent-chemically related compounds might reasonably be expected in the sample. Additionally, advanced LC–MS data processing software can be used to facilitate this task. XICs of fragment ions are then automatically extracted using a customized accurate-mass database, which includes information about the accurate mass of the target compounds and about their more significant and characteristic fragments. It is noteworthy that only compounds can be found where the modification does not affect the common fragments. In other words, if it does affect, related compounds will not be identified using this strategy.

Mass defect filtering is also feasible for the discovery of related compounds and is a common strategy for metabolite identification [Zhang *et al.* 2003]. It has also demonstrated its utility in investigating structural analogues of synthetic cannabinoids [Grabenauer *et al.* 2012]. Searching is based on the mass defect of the parent compound. While most of structural modifications result in a shift in mass, the mass defect typically remains close to that of the original compound. By applying a mass defect filter to a LC-MS data set, ions with mass defects significantly shifted from that of the parent compound can be eliminated. This process simplifies data sets and is effective at filtering out unknown related compounds from matrix elements of complex samples [Grabenauer *et al.* 2012]. One of the limitations of this approach is the need of finding an acceptable mass defect deviation that avoids losses of compounds that might be of interest, but at the same time performing an effective filtering. Another

limitation is the representativeness of the selected substance even within its own chemical family [Ibañez *et al.* 2014].

In general, common fragments and mass defect filtering can be helpful in finding drug-related compounds, such as TPs, as the number of chemically meaningful structures that can be assigned to an unknown peak is limited to structures showing a close relationship with the parent drug [Krauss *et al.* 2010]. However, minor changes in the structure of the molecule side where common fragment ions come from, could make the use of the common fragments unsuccessful for identification, or make the compounds fall out the mass defect window. To compensate for this limitation, some authors combine these two approaches [Ibañez *et al.* 2014].

Strategy 2 *– data comparison*

Another strategy is to ***compare and contrast HRMS data*** of a spiked sample (*i.e.* sample fortified with the analyte under study) and a control (blank) sample.

Under laboratory controlled conditions, natural processes such as hydrolysis and sunlight, as well as the different treatment processes applied by WWTPs such as chlorination, UV irradiation and biodegradation can be simulated. HRMS data obtained from spiked and blank samples exposed to these different degradation processes are then compared and the formed TPs can tentatively be identified and elucidated [Hernández *et al.* 2008; Quintana *et al.* 2010; Wick *et al.* 2011].

Manufacturers provide automated software algorithms to facilitate the data processing and detect TPs. Generally these algorithms are designed to compare and contrast data of the presumptive positive sample with the blank sample. In most software packages, data comparison can be performed in two ways. Firstly, for expected compounds, including known or expected transformation processes such as hydroxylation and chlorination in the processing settings. It basically consists of performing XICs of specific and expected exact masses at narrow mass window from the spiked sample and checking their absence in the blank. Secondly, for unexpected

TPs, where mass spectra of spiked versus control samples are compared. The software automatically generates XICs of a defined mass range and highlights differences due to the presence of new compounds, which are attributed in principle to transformation processes.

On the basis of the accurate mass measurements, possible elemental compositions for the peaks of interest (*i.e.* (de)protonated molecules) can be calculated (e.g. with a maximum deviation of 2 mDa). Furthermore, hybrid HRMS systems offer the possibility to obtain product ions, which are very helpful for the proposal of convincing molecular structures. Once more, it is very useful to have previous knowledge on the formation of possible fragment ions. When fragmentation pathways are less known, structures of fragment ions might alternatively be justified using specialist software such as MassFragment (Waters). Although understanding of basic fragmentation rules is still required, this potent software is a helpful tool as it allows justifying the fragment ions observed, reducing the number of candidates [Boix *et al.* 2013; Ibáñez *et al.* 2013a].

Nevertheless, one has to bear in mind that despite the amount of information obtained, an unambiguous identification is in most cases not possible by HRMS alone without an authentic reference standards or the application of complementary techniques like NMR. However, reference standards are mostly unavailable for TPs, and concentrations are often too low to perform NMR. Yet, the performance of experiments under laboratory controlled conditions in combination with hybrid HRMS is, nowadays, one of the most useful tools to identify TPs. LC-HRMS is an accepted technology for generating meaningful structure suggestions of suspects and unknowns present at low concentrations in environmental samples. Powerful software tools facilitate processing the large amount of data generated and might even perceive (low abundant) compounds overlooked by visual inspection.

4.2 Scientific articles

A detailed investigation of fragmentation pathways is important as information provided can be very helpful for data interpretation when searching for related compounds, but also for the appropriate selection of specific SRM transitions in MS/MS based analytical methods. In this chapter, accurate mass data provided by LC-QTOF MS were used to understand the fragmentation of several IDs and their metabolites. Furthermore, knowledge of common fragments in combination with accurate mass data obtained by QTOF MS in MS^E mode, and experiments performed under laboratory controlled conditions was very helpful for the elucidation of several TPs of cocaine and its main metabolite BE in water.

The first work (*scientific article 6*) presents a study of the fragmentation pathways of several classes of IDs, such as amphetamines, ATS, cocaine, opioids, cannabis, ketamine. Accurate mass spectra of the selected compounds, their metabolites and deuterated analogues were obtained using LC-QTOF MS running in both MS/MS and MS^E mode. Structures of product ions were proposed using basic fragmentation concepts and rules. In several cases, accurate mass data allowed the confirmation of structures and fragmentation pathways previously proposed in the literature based on nominal mass measurements. However, in some cases of ambiguity, HR MS allowed new insights.

In a second work (*scientific article 7*), a study on TPs of cocaine and its main metabolite BE in water has been performed. Several degradation experiments including hydrolysis, chlorination and photo-degradation (both ultraviolet irradiation and simulated sunlight), were carried out under laboratory-controlled conditions. The TPs formed were investigated by LC-QTOF MS running in MS^E mode using specialist software (Metabolynx, Waters) to facilitate this task. Analysis allowed the discovery of several TPs of cocaine and BE. Various known metabolites could be confirmed using their reference standards and information on retention time and fragment ions. Other unknown TPs could tentatively be identified, even though no standard were available. The accurate mass data, the application of basic fragmentation rules and the knowledge of the fragmentation pathways of cocaine and BE was very helpful in this elucidation process.

It is noteworthy, that *scientific article 6* and *scientific article 7* result from a close collaboration between the PhD candidate and Prof. Dr. Wilfried Niessen, a recognised specialist on mass spectrometry, of hyphen MassSpec, Leiden, the Netherlands.

The results of the two studies presented in this chapter have been published in:

- *Journal of Mass Spectrometry* 46, **2011**, 865 - 875
- *Science of the Total Environment* 443, **2013**, 200 - 208

Chapter 4.2.1, scientific article 6

Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MS^E accurate-mass spectra

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Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MS^E accurate-mass spectra

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Abstract

A study of the fragmentation pathways of several classes of drugs of abuse (cannabinoids, ketamine, amphetamine and amphetamine-type stimulants (ATS), cocaine and opiates) and their related substances has been made. The knowledge of the fragmentation is highly useful for specific fragment selection or for recognition of related compounds when developing MS-based analytical methods for the trace-level determination of these compounds in complex matrices. In this work, accurate-mass spectra of selected compounds were obtained using liquid chromatography coupled to quadrupole time-of-flight mass spectrometry, performing both MS/MS and MS^E experiments. As regards fragmentation behavior, the mass spectra of both approaches were quite similar and were useful to study the fragmentation of the drugs investigated. Accurate-mass spectra of 37 drugs of abuse and related compounds, including metabolites and deuterated analogues, were studied in this work, and structures of fragment ions were proposed. The accurate-mass data obtained allowed to confirm structures and fragmentation pathways previously proposed based on nominal mass measurements, although new insights and structure proposals were achieved in some particular cases, especially for amphetamine and ATS, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) and opiates.

Keywords

Illicit drugs of abuse, fragmentation pathways, metabolites, accurate mass, liquid chromatography, time-of-flight mass spectrometry

1. Introduction

In the recent years, there has been an increasing concern on the occurrence of drugs in the environment. In one of the first papers, Ternes *et al*. [1] reported significant concentrations of different classes of drugs in sewage and surface waters. Since then, a notable number of studies have been reported, and the number of articles published in this area is still increasing [2]. A group of drugs of particular interest are (illicit) drugs of abuse, which are frequently found in the aquatic environment, particularly in urban wastewater. Methods based on liquid chromatography-tandem mass spectrometry (LC–MS/MS) using triple quadrupole (QqQ) analyzers are important for determination of these compounds and their metabolites. This allows the rapid and efficient simultaneous quantification and confirmation at low analyte concentrations, e.g. ng/l levels, in samples such as surface water and urban wastewater with little sample manipulation [3, 4].

Wide-scope screening of organic contaminants in environmental samples is gaining popularity, thanks to the hyphenation of LC to high-resolution mass spectrometry (HR MS), e.g. orbitrap and time-of-flight (TOF) instruments. HR MS has strong potential for detection and identification purposes as a consequence of the full-spectrum acquisition with satisfactory sensitivity – allowing accurate-mass measurements of the analyte molecule and/or its main fragments –, the ability of performing retrospective analysis without the need of additional sample injections, and the feasibility of investigating a large number of contaminants after MS acquisition using a post-target approach [5]. LC–HR MS has been successfully applied for accurate-mass screening of (polar) target compounds and/or their metabolites, and to discover nontarget contaminants [6 - 10]. Some papers report on the use of LC–HR MS for identification of illicit drugs and metabolites in surface water and wastewater [11, 12]. Accurate-mass measurements of protonated molecules and their fragments allowed the reliable identification of the target compounds.

Data obtained on the levels of drugs of abuse in urban wastewater have been used to estimate illicit drugs consumption of a certain community and to appreciate their potential environmental impact [13 - 15]. To fully understand the real impact of these

compounds in the environment, not only parent drugs should be taken into account but also their metabolites. Some of them are well known to be excreted by human beings and might be observed in the aquatic environment. In addition, other potentially hazardous transformation products might be formed in the aquatic environment or in wastewater treatment plants as well. From an analytical point of view, knowledge on the fragmentation of different classes of illicit drugs is of great interest, as the recognition of specific product ions and the application of basic fragmentation rules may help to elucidate 'unknown' related compounds. The selection of more specific fragment ions would allow minimizing potential interferences and would be of great help to confirm the identity of the compounds detected in complex matrices.

When using a QTOF instrument, application of the MS^E mode is feasible, i.e. performing simultaneous acquisition of MS spectra at low and high collision energy [12]. In addition, conventional MS/MS experiments can be made to obtain accurate-mass product ion spectra, which is highly useful for confirmative purposes. Both approaches are powerful for investigation of fragmentation of drugs of abuse and have been applied in this paper.

Several papers report on fragmentation of drugs of abuse, making use of GC–MS [16, 17] and/or LC–MS with different analyzers. Only LC–MS was taken into account in this paper as fragmentation of molecular ions generated by electron ionization, typically applied in GC–MS, notably differs from fragmentation of protonated molecules generated by atmospheric pressure ionization typically employed in LC–MS. LC–MS is nowadays the technique of choice for the wide majority of drugs of abuse and metabolites.

Mass analyzers, acquiring in nominal or accurate mass, have been used to the study of fragmentation of drugs of abuse. Recently, Castiglioni *et al*. [3] reviewed mass spectra and fragmentation patterns of several classes of illicit drugs. In most cases, low-resolution MS analyzers have been applied to elucidate the most abundant fragments [18 - 22]. However, for more detailed fragmentation and in cases of ambiguity, HR MS or

a combination of techniques (including the use of isotope labeled analogues) needs to be applied [23 - 27]. In this paper, we report a detailed study on the fragmentation of several classes of illicit drugs and their related substances (metabolites/degradation products) using accurate-mass measurements provided by LC–QTOF MS. Whereas some information already exists in the literature, often data are yet incomplete, or, in some cases, the interpretation of mass spectra might be questionable. Information provided in this work will be of help for future method development and data interpretation in LC–MS-based analytical methodology.

2. Experimental

2.1. Reagents and chemicals

Illicit drugs and metabolites studied were the following: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-methamphetamine (MDMA or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), R(+)-methcathinone, 1R,2S(−)ephedrine, cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, norcocaine, ecgonine, ketamine, heroin, codeine, norcodeine, morphine, 6-monoacetylmorphine (6-MAM), normorphine and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH). These compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), Cerilliant (Round Rock, TX, USA) and the National Measurement Institute (Pymble, Australia) as solutions in methanol (MeOH), acetonitrile or as salt. Standard stock solutions of each compound were prepared at 100 mg/l in MeOH or acetonitrile.

Deuterium-labeled compounds were all obtained from Cerilliant as solutions in MeOH or acetonitrile at a concentration of 100 mg/l: amphetamine-d_6, methamphetamine-d_5 MDA-d_5, MDMA-d_5, MDEA-d_5, 1S,2R(+)ephedrine-d_3, cocaine-d_3, cocaethylene-d_8, benzoylecgonine-d_3, ecgonine-d_3, ketamine-d_4, heroin-d_9, codeine-d_6, morphine-d_3, 6-MAM-d_6 and THC-COOH-d_3.

Intermediate solutions (10 mg/l) were prepared by diluting the stock solutions with MeOH. All standard solutions were stored in amber glass bottles at −20 °C. Working

solutions of individual standards were prepared at a concentration of 100 µg/l in MeOH:water (5:95, v/v) just before MS^E and MS/MS experiments.

Chemical structure, exact mass of the protonated molecule and CAS number of the selected illicit drugs and their deuterated analogues are listed in Table SI 1 of Supporting Information (SI).

2.2. Instrumentation

A Waters Acquity ultra-high-performance liquid chromatography system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (QTOF Premier, Waters Micromass, Manchester, UK) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operated in positive-ion mode.

Two types of acquisition, MS/MS and MS^E, were performed. For MS/MS experiments, cone voltage and collision energy ramp were optimized for each compound individually (Table SI 2). For MS^E experiments, two acquisition functions with different collision energies were created: the low energy function (LE), and the high energy (HE) function, with the same compound-dependent optimized cone voltage and collision energy ramp as for MS/MS.

Further details on instrument operating conditions both chromatographic and spectrometric can be found elsewhere [12].

3. Results and discussion

3.1. General aspects

Several papers report on MS fragmentation of drugs of abuse. Recently, Castiglioni *et al*. [3] published an excellent review on fragmentation of several drugs of abuse and metabolites frequently found in wastewater and surface water, based on nominal mass measurements by LC–MS/MS [triple quadrupole (QqQ)]. A more detailed investigation of the fragmentation should be based on accurate-mass data. This would allow the elucidation of the chemical formulae of fragments with higher confidence. The

information obtained would permit a more rational selection of structural specific fragments, which is of interest for LC–MS analytical methods, e.g. using TOF or QqQ mass analyzers, giving a more reliable identification of analytes and minimizing the possibility of reporting false positives or false negatives. A reduction of interferences and an increase in the signal-to-noise ratio could also be obtained by performing extracted ion chromatograms (XIC) from full-spectrum accurate-mass data, selecting narrow *m/z* windows (e.g. ± 0.01 Da) [28, 29]. In analyses involving selected reaction monitoring (SRM) in a QqQ instrument, the same effect could be expected by selection of more specific ions.

For the present paper, data were acquired in both MS/MS and MS^E mode using a QTOF instrument in positive-ion ESI. The MS^E acquisition strategy was recently introduced for QTOF instruments [12, 30 - 32]: a continuous scan-wise switching is made between low collision energy, to detect ions from intact molecules, and high collision energy, to acquire fragmentation data. This technique enables acquisition of ions from parent molecules, their isotopic patterns and their fragment ions in a single injection. It has become a powerful tool for wide-scope screening of a large number of compounds with strong identification capabilities.

Although product ion and MS^E mass spectra were highly comparable, the different characteristics of each mode were useful for the elucidation of certain fragments. When applied to samples, e.g. urban wastewater, product ion mass spectra are generally cleaner and the sensitivity is higher, as specific precursor ions are selected in the quadrupole.

Optimum MS/MS and (HE) MS^E conditions for each compound were established and are listed in Table SI 2 of SI. Data interpretation was based on accurate *m/z* values observed for the fragment ions, often both of the drugs and of a deuterated analogue, the molecular formulae derived from the measured accurate *m/z* and the general concept that fragmentation should involve logical neutral losses and no major rearrangements in the structure of the drug. Based on these concepts, structures for the fragment ions have been proposed. No attempts were made to prove the proposed

structures by further experiments or theoretical calculations. Isotope information from the MS^E was also of help. Accurate-mass information allowed us to confirm the fragment ion identity previously proposed by other authors, e.g. based on nominal mass measurements. In addition, accurate-mass data were essential to understand the fragmentation and/or to discard structures suggested in the literature.

3.2. Cannabinoids

The most psychoactive cannabinoid is Δ^9-tetrahydrocannabinol (THC). The amount of THC is used as a measure of 'cannabis potency'. However, as marker compounds of cannabis consumption, the THC metabolite 11-nor-9-carboxy-Δ^9-THC (THC-COOH) and its glucuronic-acid conjugate (THC-COOH-glucuronide) are generally analyzed by LC–MS/MS in human plasma [18] and in urine samples [33, 34]. In wastewater, where glucuronide conjugates are easily hydrolyzed to the free acid by beta-glucuronidase of fecal bacteria [35], THC-COOH is the analytical target of choice. THC-COOH has been determined by LC–MS/MS both in negative-ion [36, 37] and positive-ion mode [38, 39]. The most abundant fragment ions in the product ion mass spectra of the deprotonated and protonated molecule of THC-COOH have been discussed before [3, 18]. A structure of a less abundant, but more specific fragment ion, with *m*/z 193, to be (2,6-dihydroxy-4-pentylphenyl)methylium was proposed by Maralikova *et al*. based on QqQ data [18].

The proposed fragments of the protonated molecule could be confirmed by our accurate-mass data. Additionally, with the help of the product ion mass spectra (Fig. 1B and C) of the protonated THC-COOH ion and THC-COOH-d_3 deuterated analogue, several less abundant fragment ions, not reported previously, could be identified (Fig. 1A). As the D_3C group is at the end of the pentyl chain, any +3 shift between the *m*/z values in the product ion mass spectra of the $[M + H]^+$ ion of THC-COOH and its deuterated analogue indicates that the pentyl chain is unaffected by the fragmentation. This is true for the *m*/z 193 fragment ion, but also for *m*/z 257, corresponding to the loss of propene (C_3H_6) from the fragment ion *m*/z 299 $[M + H–HCOOH]^+$, which involves ring opening of the saturated six-member ring. On the other hand, the fragment ions with *m*/z 229 and 187 do not show the +3 shift, indicating that

these fragments originate from the loss of pentene (C_5H_{10}) and the combined loss of pentene and propene, respectively, from the fragment ion with *m/z* 299.

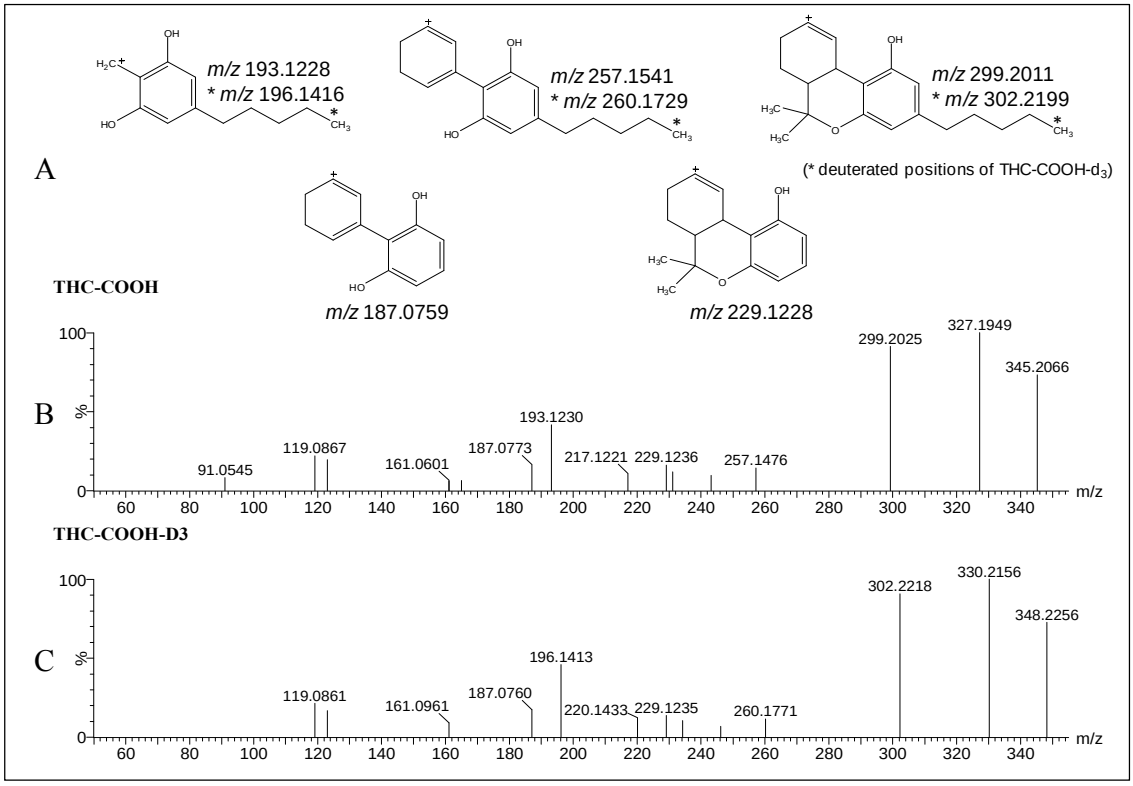

Figure 1. Structure proposals for some THC-COOH fragment ions (A), product ion (QTOF) mass spectra of $[M+H]^+$ of THC-COOH (B) and THC-COOH-d_3 (C).

In the case of THC-COOH, accurate-mass information served as a confirmation tool of fragments previously suggested based on nominal mass measurements. Comparison of mass spectra of this metabolite and of its deuterated analogue was essential for the identification of less abundant fragments and understanding the fragmentation route.

3.3. Ketamine

In the aqueous environment, detection of ketamine in wastewater has been reported a few times [40, 41]. Ketamine is a licit pharmaceutical, and as such, has some abuse potential and is connected to the 'club drug' scene [42].

As shown in prior work by the authors, the MS^E spectrum of the $[M+H]^+$ ion and fragment ions of ketamine showed useful isotopic information related to the presence of one chlorine atom [40]. This information together with the accurate mass of both precursor and fragment ions confirmed the fragmentation proposed by Wang *et al*. [19] with high confidence. The chlorine atom is still present in all fragments, with the exception of those with *m*/z 115 and 116. Most of them can be explained being due to the sequence of losses of water and methylamine (in either order) to the ion *m*/z 189, with intermediate ions *m*/z 220 and 207, respectively, and further fragmentation to ions *m*/z 179 (CO loss from the ion with *m*/z 207) and *m*/z 125. Most likely, the (2-chlorophenyl)methylium ion with *m*/z 125 is more readily formed from the ion *m*/z 179, due to an inductive cleavage involving the loss of C_4H_6, because the alternative route from *m*/z 189 requires the cleavage of two C–C bonds, which seems less likely (Fig. SI 1).

The ions with *m*/z 115 and 116 ($C_9H_7^+$ and $C_9H_8^{+\bullet}$, respectively) involve the loss of chlorine, either as HCl or as a radical. From the ketamine-d_4 spectrum (Fig. SI 1B), one may conclude that this may be a Cl-radical, HCl or DCl. However, the pathway involved in the loss of chlorine is certainly not evident, because for instance an ion with *m*/z 151/153 is not clearly observed.

The fragmentation of ketamine derivatized heptafluorobutyric anhydride has been studied by Pieri *et al*. [17] using GC–MS and electron ionization. Obviously, a fragmentation pathway of the derivatized ketamine under these conditions significantly differs from that reported here.

3.4. Amphetamine and amphetamine-type stimulants

Since Jones-Lepp *et al*. reported methamphetamine and MDMA in effluent wastewater in the USA [43], amphetamine and amphetamine-type stimulants (ATS) have frequently been detected at ng/l level in influent wastewater and sometimes in effluent samples [38, 41, 44 - 46]. ATS are a group of synthetic stimulants, including predominantly amphetamine, methamphetamine, MDA, MDMA (ecstasy) and MDEA. Amphetamine analogues, such as methcathinone, may also be included in this group. Furthermore, the most commonly used ATS chemical precursors such as ephedrine fall under

international control, and their seizure can provide some limited indications about manufacturer trends [42].

The MS/MS fragmentation patterns of the $[M + H]^+$ ion of amphetamine (Fig. 2A) and methamphetamine are straightforward with the fragment ion *m*/z 119, resulting from the loss of ammonia or methylamine, respectively, the tropylium ion ($C_7H_7^+$ with *m*/z 91) due to a β-C-C cleavage, and the secondary fragment *m*/z 65 ($C_5H_5^+$) due to the loss of acetylene from the ion *m*/z 91, as common fragment ions. The product ion mass spectrum of amphetamine-d_6 leads to some interesting novel observations (Fig. 2B). Given the deuterium positions in the amphetamine-d_6 (C_6H_5–CD_2–$CD(-NH_2)$–CD_3), a straightforward β-C-C cleavage would lead to a tropylium ion with the formula $C_7H_5D_2^+$ (*m*/z 93). However, next to the ion with *m*/z 93, two other ions were observed consistent with *m*/z 94 ($C_7H_4D_3^+$) and *m*/z 95 ($C_7H_3D_4^+$), which indicate scrambling of the labels during fragmentation (Fig. 2C). NMR spectroscopy was applied to confirm the specified positions of the D-labels. In turn, the ions *m*/z 93, 94 and 95 provide secondary fragmentation involving the loss of either HCCH, HCCD, or DCCD, leading to fragment ions with *m*/z 65 ($C_5H_5^+$), 66 ($C_5H_4D^+$), 67 ($C_5H_3D_2^+$), 68 ($C_5H_2D_3^+$) and 69 ($C_5HD_4^+$) (Fig. 2D). Essentially, the same results were obtained by collision-cell fragmentation of the precursor ions *m*/z 93, 94 and 95, generated by in-source fragmentation of amphetamine-d_6. In our opinion, these observations are most readily explained if the fragment ion *m*/z 91 has a tropylium ion and not a benzyl cation structure. Moreover, it puts some light on the complexity of the fragmentation mechanism of a protonated molecule, as the β-C-C cleavage apparently involves scrambling of ring and chain hydrogen atoms. Further complexity is added by the product ion mass spectrum of methamphetamine-d_5, where next to the most abundant ion *m*/z 92 ($C_7H_6D^+$), consistent with a straightforward β-C-C cleavage, the ions *m*/z 91 (one D-label lost, $C_7H_7^+$) and *m*/z 93 (one D-label gained, $C_7H_5D_2^+$) are also observed. Similar secondary fragmentation was obtained, involving losses of HCCH, HCCD and DCCD.

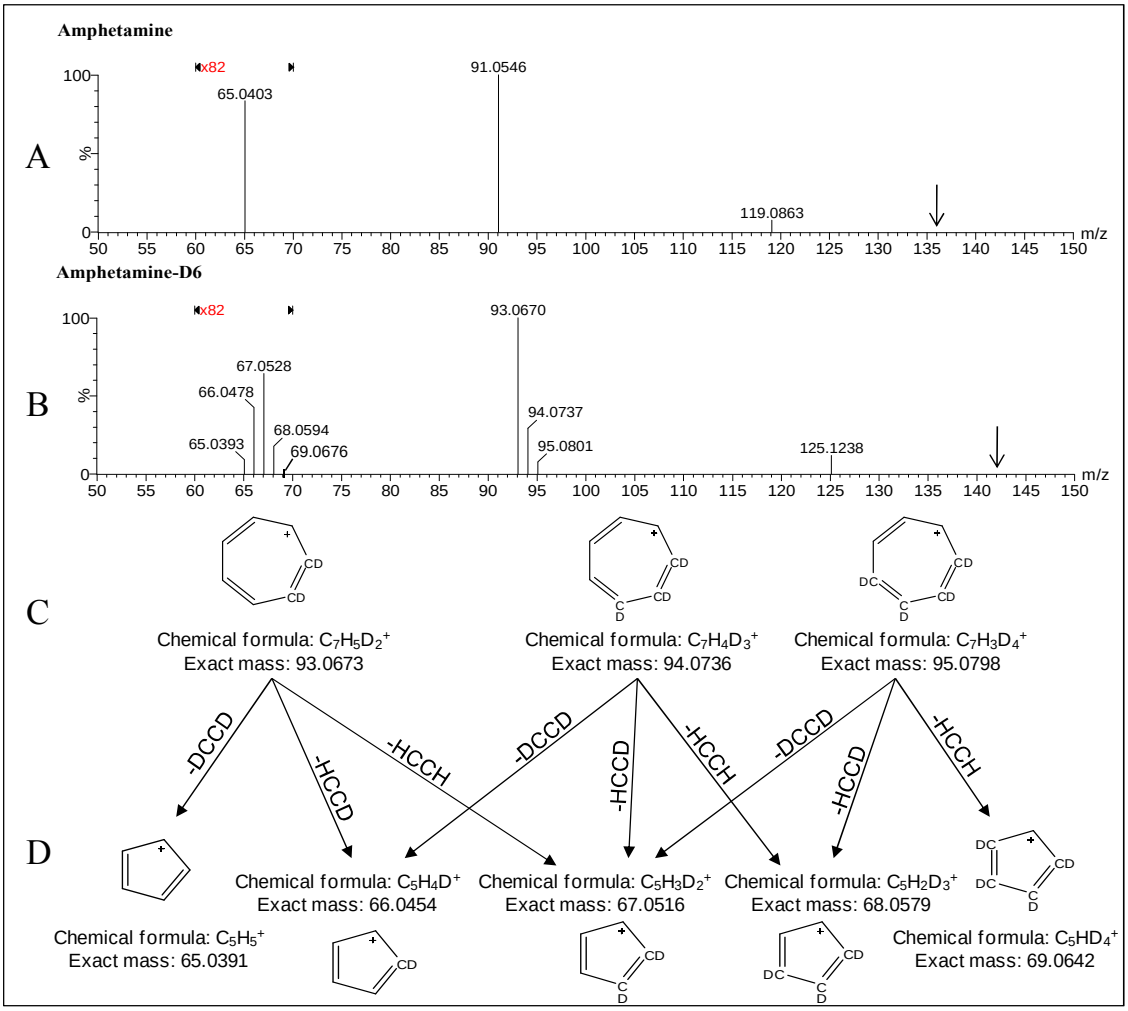

Figure 2. Product ion (QTOF) mass spectra $[M+H]^+$ of amphetamine (A) and amphetamine-d_6 (B) (range *m/z* 60-70, 82x magnified), H/D scrambling of the tropylium ion (C) and the secondary fragments of the tropylium ion (D) of amphetamine-d_6.

Compared to other amphetamine analogues, methcathinone and ephedrine have been scarcely studied. To the best of our knowledge, a detailed study on their fragmentation pathway has not been reported. The structures of these compounds are comparable to methamphetamine, yet with either a ketone- or hydroxyl-group at the C^1 position, respectively. This significantly affects the fragmentation. Methcathinone

([M + H]$^+$ with *m*/z 164) (Fig. 3A) and ephedrine ([M + H]$^+$ with *m*/z 166) (Fig. 3B) both show the loss of water with remarkable ease from the keto- or hydroxyl-function, respectively, and subsequently the loss of $CH_3^{\bullet}$, resulting in the radical cations *m*/z 131 and *m*/z 133, respectively. To this end, there are two methyl groups that could be lost: N–CH_3 and C^2–CH_3. From the ephedrine-d_3 spectrum (Fig. 3C), one can deduce, that both losses actually occur, with the loss from C^2–CH_3 resulting in a more abundant fragment (*m*/z 136) than the loss from N–CH_3. The loss of CH_4 is also observed, leading to fragments with *m*/z 130 and 132 of the unlabeled compounds, respectively. Structures for these fragment ions are proposed in Fig. 3. At low *m*/z, the spectra differ considerably (Fig. 3A and B). Methcathinone shows a fragment with *m*/z 105.0699. Accurate-mass data (Fig. 3A) prove that this ion corresponds to C_6H_5–C^+H–CH_3 (calculated *m*/z 105.0704, mass error 0.5 mDa) rather than the expected C_6H_5–C ≡ O^+ (calculated *m*/z 105.0340, mass error 35.9 mDa). Ephedrine, on the other hand, shows a fragment with *m*/z 117, owing to subsequent losses of water and methylamine, as well as a fragment with *m*/z 115, which can be written as a stable ring structure with a resonance-delocalized charge, after an apparently easy loss of H_2 from the ion with *m*/z 117.

MDA, MDMA and MDEA, containing a dioxole ring, all give similar fragmentation patterns, with the exception of some minor fragments at the lower end of the spectra and, obviously, the protonated molecule. As an example, MDA ([M + H]$^+$ with *m*/z 180) (Fig. 4A) shows a loss of NH_3 to a fragment ion with *m*/z 163 and a β-C-C cleavage leading to a 1,3-benzodioxol-5-ylmethylium ion with *m*/z 135 due to the loss of ethylamine. Structure of these and some other fragment ions are proposed in Fig. 4. This pattern is similar to what is observed for amphetamine and related compounds, as described above. With MDMA and MDEA, the same β-C-C cleavage also leads to minor immonium ions with *m*/z 58 and 72, consistent with the loss of 5-methyl-1,3-benzodioxole from [M + H]$^+$. The fragment ion with *m*/z 133 can be considered as a secondary fragment of the ion with *m*/z 163 (after loss of ammonia) and corresponds to a loss of $H_2C = O$ due to cleavage of the methylenedioxy ring. An interesting fragment is the ion with *m*/z 105, since this ion has been interpreted as being due to the loss of $H_2C = O$ from the ion with *m*/z 135 (thus with formula $C_7H_5O^+$, calculated *m*/z 105.0340)

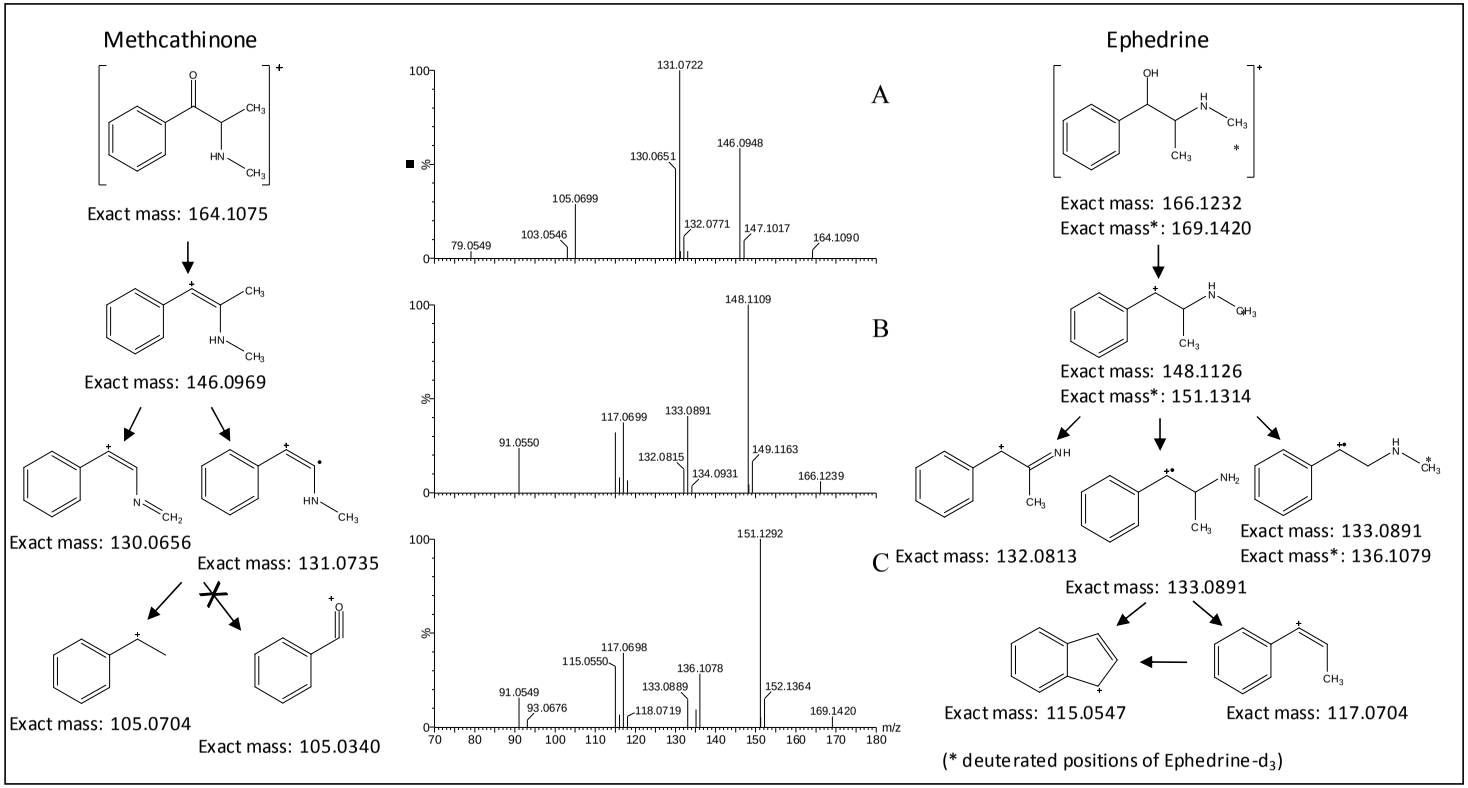

Figure 3. Structure proposals for some of the fragment ions in the product ion mass spectra of $[M+H]^+$ of methcathinone (left) and ephedrine (right). MS^E (HE) spectra (middle) of methcathinone (A), ephedrine (B) and ephedrine-d_3 (C).

[3], whereas our accurate-mass data (*m*/z 105.0703) suggests that the correct formula is $C_8H_9^+$ (calculated *m*/z 105.0704, mass error 0.1 mDa). Structures of both $C_7H_5O^+$ and $C_8H_9^+$ are shown in Fig. 4. In addition, data of its labeled analogue (Fig. 4B) proofs this interpretation of *m*/z 105.0704 to be correct, as it would be the only way to account for five labels in this fragment.

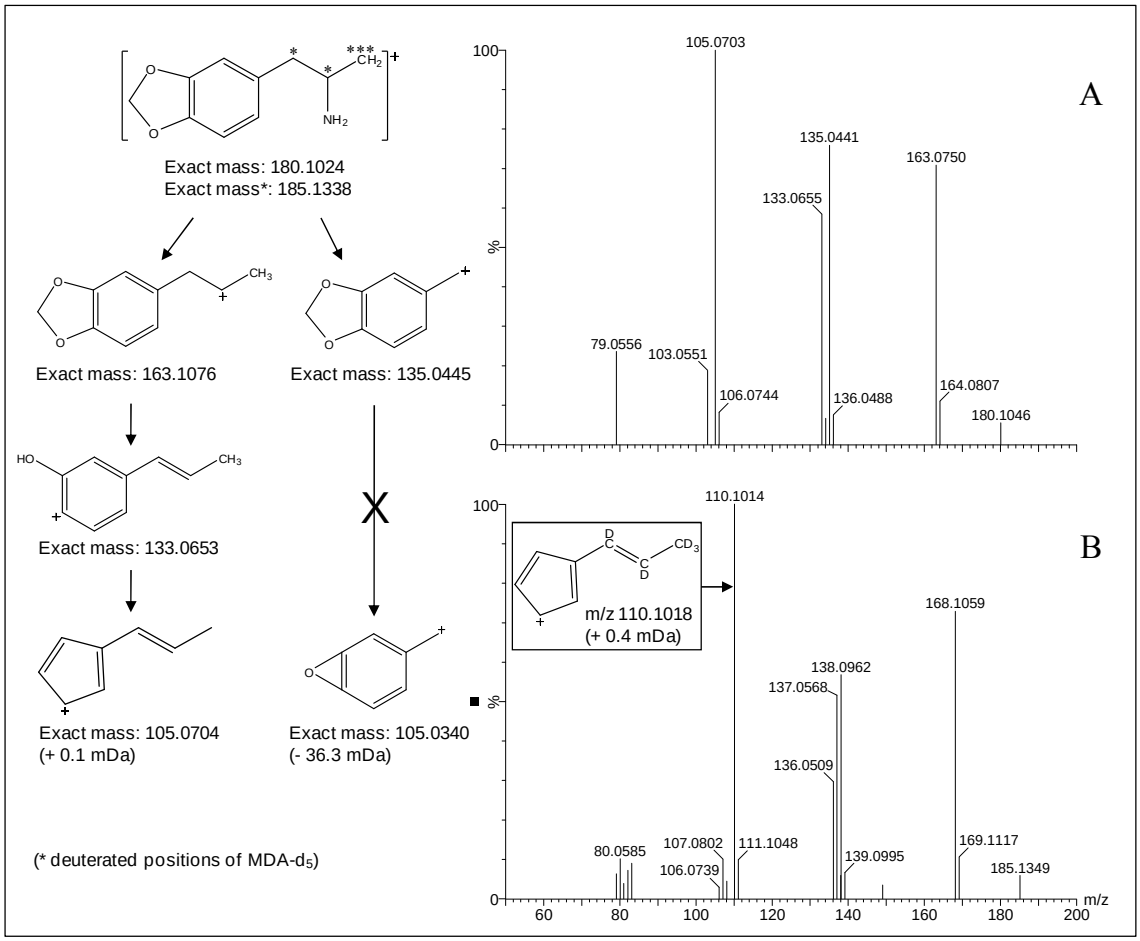

Figure 4. Structure proposals for some of the fragment ions of $[M+H]^+$ of MDA (A), MDA-d_5 (B): insert, structure of MDA deuterated fragment *m*/z 110.

Study of the labeled analogues of MDA, MDMA and MDEA was consistent with our expectation. Some H/D scrambling was observed, similar to the observations of the

labeled analogues of (meth)amphetamine. No H/D scrambling was observed in the spectra of MDEA-d_5, where D-labeling is at the N-ethyl group.

3.5. Cocaine

Cocaine and its metabolites are frequently reported to be found in the aquatic environment. Concentrations are relatively high (sometimes up to µg/l level), especially for its major metabolite benzoylecgonine, compared to other illicit drugs [41, 44, 45].

By using product ion mass spectra of labeled and unlabeled compounds, Wang *et al*. [20] comprehensively described fragmentation pathways for cocaine, its metabolites and pyrolytic degradation products. Later, Castiglioni *et al*. [3] also reported fragmentation data on this group of compounds, supporting them with the corresponding mass spectra. The fragmentation mechanisms proposed by these authors are confirmed by our accurate-mass spectra (data not shown). The most abundant fragment of cocaine and most of its metabolites can be assigned to the neutral loss of benzoic acid (122 Da). The remaining fragment ions can be considered as subsequent fragmentation of the resulting ion $[M + H - 122]^+$, involving a further loss of methanol or water.

3.6. Opiates

As they are frequently used as narcotic to relief pain, opiates can enter the environment not only by way of drug abuse but also due to clinical usage. The opiates most widely used as abusive drug are heroin, morphine and codeine. These compounds have been found (few ng/l) in wastewater, together with their related metabolites, 6-monoacetylmorphine (6-MAM) and norcodeine [36, 37, 39]. Glucuronide conjugates such as morphine-3-β-glucuronide or morphine-6-β-glucuronide have also been found in sewage water [36]. Fragmentation of these conjugates shows an intense fragment ion corresponding to the characteristic loss of the dehydroglucuronic acid group (loss of 176 Da) [3]. Here, only the fragmentation of unconjugated opiates was studied.

The structures of the opiates and their deuterated analogues studied in this work are illustrated in the SI (Table SI 1). In addition to the mentioned references, visual assistance is available to the reader (Fig. SI 2) to get more insight in the proposed fragmentation route of opiates. Fragmentation pathways at the high end of the spectra have been studied by other authors [23-25], and our accurate-mass data confirm the earlier interpretation. However, fragment ions at the lower end of the spectra have generally been ignored.

The loss of the ketene $H_2C=C=O$ and acetic acid (from heroin), acetic acid (from 6-MAM) and water (from morphine) in all three cases results in a fragment ion at *m*/z 268 ($C_{17}H_{18}NO_2^+$) (see Fig. SI 2). Subsequent fragmentation involves cleavages in the morphinan backbone. This results in complex fragmentation patterns, in which heroin, 6-MAM and morphine, but also nor-morphine, codeine and nor-codeine, show many similar features.

Taking heroin as an example (Fig. 5A), an initial cleavage of the piperidine ring occurs. The most abundant fragment results from the loss of $H_3C–CH_2=N–CH_3$ (57 Da) to fragment ion with *m*/z 211 ($C_{14}H_{11}O_2^+$), although fragment ions due to the loss of $H_2C=N–CH_3$ (43 Da) to *m*/z 225 ($C_{15}H_{13}O_2^+$) and $H_2N–CH_3$ (31 Da) to *m*/z 237 ($C_{16}H_{13}O_2^+$) are also observed. The structure proposed by Zhang *et al*. [25] for the ion with *m*/z 225, involving a seven-member ring, seems unlikely, as it would require significant rearrangement of the structure; an alternative proposal is given in Fig. 5 (top).

Next to the ion with *m*/z 211, an ion with *m*/z 209 ($C_{14}H_9O_2^+$) is observed, which would involve an additional double bond in the remaining structure with four fused rings. Similar behavior is observed for the fragment ions with *m*/z 193 ($C_{14}H_9O^+$) and 183 ($C_{13}H_{11}O^+$), which are due to losses of water or CO, respectively, from the ion with *m*/z 211; thus, ions with *m*/z 191 ($C_{14}H_7O^+$) and 181 ($C_{13}H_9O^+$) are observed as well (see Fig. SI 2). The lower *m*/z satellite peaks (*m*/z 191 and 181) show greater aromaticity, which may be the driving force in their formation.

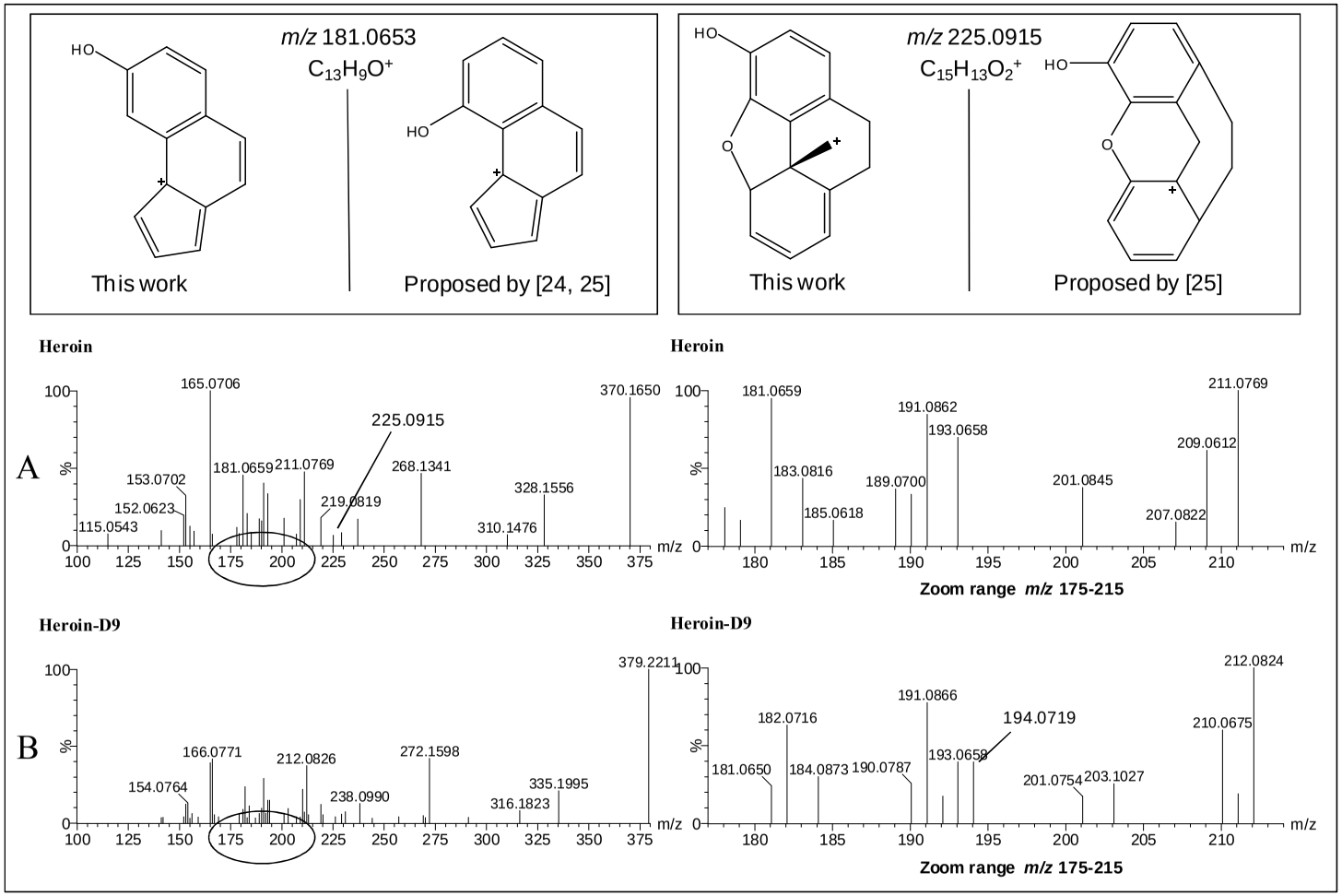

Figure 5. Product ion mass spectra of $[M+H]^+$ of heroin (A) and heroin-d_9 (B) (bottom) with zoom in the range *m/z* 175 – 215. Proposed structures for product ions *m/z* 181 ($C_{13}H_9O^+$) and *m/z* 225 ($C_{15}H13O_2^+$), involving cleavage in the morphinan backbone (top).

Careful comparison of the spectra of heroin and heroin-d_9 reveals that the loss of water to give *m*/z 193 from ion with *m*/z 211 (Fig. 5A) involves either the oxygen from 4,5-epoxide or the 3-hydroxy group, whereas the loss of CO mainly appears to involve the oxygen from 4,5-epoxide. It should be pointed out that in the heroin-d_9 spectrum the loss of water from *m*/z 212 would lead to both fragment ions with *m*/z 193 and *m*/z 194 (Fig. 5B). This is only possible if the loss of water involves the oxygen of either of the two groups, with the loss of the hydroxy-O (as HDO) leading to *m*/z 193 and the loss of the epoxy-O (as H_2O) to *m*/z 194. In heroin-d_9, the three methyl groups of heroin are CD_3 groups. In the fragment ion with *m*/z 212, two CD_3 groups have been lost with the loss of acetic acid and the N–CD_3 substituent; one D-label is still present, resulting from D-rearrangement upon the loss of the ketene $D_2C = C = O$.

Regarding the CO loss, only the fragment ion *m*/z 184 is observed in the heroin-d_9 spectrum, but not the *m*/z 183 (Fig. 5B), suggesting that only 4,5-epoxide is involved in this loss, maintaining the 3-hydroxy group. Moreover, an abundant fragment (*m*/z 182) was also observed, due to H_2 loss. This demonstrates that D-labeling of heroin and related compounds was important in elucidation of the fragmentation pathway. From our point of view, the structure suggested by others for the fragment ion *m*/z 181 of heroin and related compounds, with a 4-hydroxy group, is unlikely [24, 25]. We think that the 3-hydroxy group is more appropriate (Fig. 5 (top)).

An abundant fragment in the spectra of opiates is the ion *m*/z 165. It is frequently selected in SRM analysis for quantification or confirmation of opiates in wastewater [37, 39]. The ion *m*/z 165/166 can be derived from the ion *m*/z 193/194 due to the loss of CO or from the ion *m*/z 183/184 due to the loss of water.

It is noteworthy that the fragment ions involving additional loss of H_2 and thus the formation of a double bond (*m*/z 191, 181 and also 153, see below) as well as the most abundant ion in the quadrupole spectra (*m*/z 165) were not observed in ion-trap MS^2 spectra, but only as minor ions in subsequent MS^3 and MS^4 spectra [23, 25].

Fragment ions at the lower end of the spectra have been generally ignored. The formation of ions such as *m*/z 157, 141 and 115 is complex and involves multiple neutral

losses and/or losses of larger neutral fragments (Fig. 5A). Structure elucidation is complicated, and unambiguous structures cannot always be proposed. At this point, it was helpful to perform additional experiments using a triple-quadrupole MS/MS instrument. By performing precursor-ion scans of the more abundant low-*m*/z fragment ions, more insight was obtained in their fragmentation pathways.

The precursor ions of the morphine product ion with *m*/z 157 ($C_{11}H_9O^+$) are the *m*/z 185, 201 and 229 ions, involving neutral losses of CO, $H_3C–CH=O$ and $CO + H_3C–CH=O$, respectively. We compared these data with the precursor-ion scan data of the related product ion of codeine, i.e. *m*/z 171. Codeine contains a methoxy- instead of a hydroxy-group on the C^3 position resulting in a mass shift of +14 in the *m*/z value of various product ions relative to morphine. Furthermore, the deuterated analogue of codeine (codeine-d_6) contains three additional D-labels on this methoxy-group compared to morphine-d_3. Thus, the corresponding codeine fragment ion with *m*/z 171 results from precursors *m*/z 199, 215 and 243; the mass shift of 14 Da indicates an identical fragmentation pathway in which the methoxy-group is kept.

Based on these results, possible fragmentation pathways for the low-*m*/z fragments are discussed in more detail starting from the product ions with *m*/z 243 and 229 of codeine and morphine, respectively, formed by the complete loss of the piperidine ring (see Fig. SI 2).

The formation and structure of fragment ions *m*/z 141 ($C_{11}H_9^+$) and *m*/z 115 ($C_9H_7^+$) are less evident. Results from precursor-ion scans for *m*/z 141 showed the presence of *m*/z 201 and 229 for morphine, and with *m*/z 215 and 243 for codeine, pointing out an identical fragmentation route, retaining the oxygen-group at the C^3 position. A key role can be attributed to the precursor ions *m*/z 201 (morphine) and *m*/z 215 (codeine), which can be formed by the loss of CO from the *m*/z 229 and *m*/z 243 ions, respectively. However, the loss of CO can be associated with the oxygen either from the 4,5-epoxide or the 6-hydroxy group. In Fig. 6 (top), three possible structures for fragment ion *m*/z 201 (and *m*/z 215) are proposed: A and B involving the loss of 6-hydroxy oxygen, whereas C involves the loss of 4,5-epoxide group. Structures A and B are convincing to explain the

formation of the ion *m*/z 141 from the precursor ion *m*/z 201 (or *m*/z 215). It appears to correspond to a one-step loss of HC(=O)–CH_2OH for morphine and of HC(=O)–CH_2OCH_3 for codeine. Structure C is unlikely to be involved in the formation of *m*/z 141.

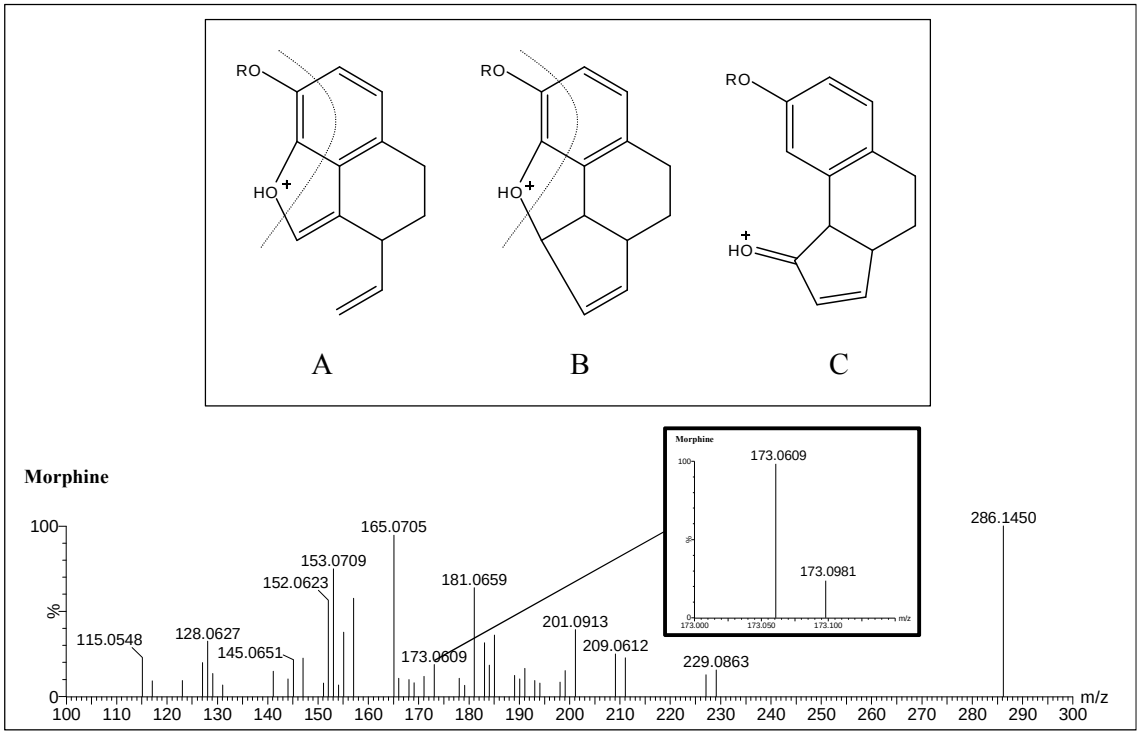

Figure 6. Product ion mass spectrum of $[M+H]^+$ of Morphine and three possible chemical structures (A, B, C) suggested for morphine fragment *m*/z 201.0915 (R = H, $C_{13}H_{13}O_2^+$) and codeine fragment *m*/z 215.0915 (R = CH_3, $C_{14}H_{15}O_2^+$). The loss of HC(=O)-CH_2OR to 141.0704 is illustrated as a dotted line (top).

Regarding fragment ion *m*/z 115, the precursor ion with *m*/z 201 (and *m*/z 215 for codeine) possibly also plays an important role in the formation of this fragment, although in this case an intermediate ion *m*/z 173 (and *m*/z 187 for codeine) is also participating in the fragmentation pathway based on precursor ion scan data. According to the high-resolution accurate-mass data (Fig. 6 (bottom)), two isobaric fragment ions with *m*/z 173 ($C_{11}H_9O_2^+$ or $C_{12}H_{13}O^+$) are formed. Zhang *et al*. proposed two different structures for this fragment ion, involving a one-step or two-step

fragmentation from *m*/z 229. Our TOF MS data clearly shows the presence of both these fragment ions, *m*/z 173.0609 and *m*/z 173.0981, demonstrating that both pathways are surely involved. However, we believe that fragment ions are formed only from the precursor ion *m*/z 201, involving a one-step loss of either C_2H_4 from structure 6A (supported by the presence of *m*/z 173.0609) or a loss of CO from structure 6 C (supported by *m*/z 173.0981).

In both cases, a further one-step fragmentation to *m*/z 115 is complicated, implying less logic rearrangements and fragmentation of aromatic rings. Identical complicated one-step fragmentation is involved in the formation of *m*/z 115 from the codeine intermediate ions *m*/z 187 ($C_{13}H_{15}O^+$ or $C_{12}H_{11}O_2^+$). Therefore, unequivocal structure for the fragment ion with *m*/z 115 could not be suggested.

3.7. Applied issues

LC–QTOF MS is a powerful technique for identification and confirmation purposes, very attractive for wide-scope screening of drugs of abuse in the environment. To take full profit of its qualitative and elucidative potential, a detailed knowledge of the fragmentation pathways is required. The possibility of searching for drug-related compounds is one of the strong points of TOF MS. Assuming that most of these compounds share similar fragmentation pathway with the parent drug, the knowledge of fragment ions of the main drugs of abuse is essential to search for metabolites, transformation and/or degradation products. Different approaches can be applied based on accurate-mass measurements of common fragment ions. Nowadays, manufacturers provide automated software algorithms to detect expected and unexpected metabolites. Most of these algorithms are designed to compare and contrast chromatograms of a presumptive positive sample with a control or blank sample. However, in environmental analysis, a representative blank sample is practically impossible to obtain, and this approach seems less useful. An alternative way is to obtain narrow-mass window XIC (e.g. ± 0.01 Da) for specific fragment ions from the HE and/or LE functions of MS^E data. When these chromatograms show additional chromatographic peaks at retention times different to that of the parent drug, the presence of parent-chemically related compounds can reasonably be

expected in the sample. This approach, based on assuming similar fragmentation pathways for parent analyte and their metabolites, has successfully been applied for investigation of pesticide metabolites [47]. Another example illustrating the relevance of knowing the fragmentation in detail is the possibility to differentiate isomers from their specific fragments, as although sharing the same empirical formula and exact mass they can suffer different fragmentation [12, 40].

The selection of specific fragment ions is not only relevant when searching for metabolites or transformation products using accurate-mass data, but also for the selection of appropriate (selective) SRM transitions in quantitative analyses using QqQ instruments. As pointed out by Pozo *et al*. [48], nonspecific transitions such as those involving the loss of water or CO may result in reporting false positives or false negatives, as the probability of finding interferences sharing the same transition increases. This is more likely to occur when dealing with analytes at sub-ppb levels in complex matrices, e.g. the determination of drugs of abuse in urban wastewater. In addition, the most sensitive transitions (typically selected in quantitative methods) not necessarily are the most selective ones. Therefore, the selection of fragment ions taking into account not only their abundance but also their specificity is of great relevance for trace-level analysis. The European Union established the acquisition of at least two SRM transitions in low-resolution MS instruments, together with the measurement of their intensity ratio for confirmation of contaminants in samples of animal origin [49]. This criterion is also frequently used in the determination of organic contaminants in environmental samples. However, no requirements are given on the selectivity/specificity of the selected ions. In our opinion, more attention needs to be paid to the specificity of the (fragment) ions in order to minimize the possibility of reporting false positives, especially when legal or societal implications are involved. An interference sharing one of the transitions may lead to report the sample as negative because of the noncompliance of the Q/q ratio, even in cases where the analyte is present. In any case, the maximum tolerances established for Q/q ratio deviations are subject of controversy, especially in complex-matrix samples [48, 50]. Unfortunately, information on Q/q ratio values in samples and reference standards is not always given in the literature. A detailed knowledge of fragmentation pathways and the expertise of the researcher on MS are

among other aspects of great importance to obtain reliable data in LC/MS-based methods.

4. Conclusions

Accurate-mass spectral data for some classes of illicit drugs and their related substances (metabolites, degradation products), which are frequently detected in environmental and wastewaters, have been obtained using QTOF MS in both MS/MS and MS^E mode. Based on these data, fragmentation pathways have been carefully elucidated. Although nominal-mass measurement can already be useful for elucidation purposes in many cases, accurate-mass information is imperative for confirmation of the identity of such fragment ions as well as to allow elucidation of structures of several important key ions. In some cases, accurate-mass data of deuterated analogues also played a key role in the elucidation of the fragmentation. All information provided by QTOF MS helped us to elucidate structures with high reliability and to complete fragmentation pathways.

The information in this paper can help the reader to interpret related spectra and to select specific fragments for the safe identification of these emerging contaminants in environmental, food or biological sample matrices.

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Supporting information

In this section, a table with useful information for 37 drugs of abuse, their metabolites and deuterated analogues investigated is shown (Table SI 1). Chemical structures, exact masses of the protonated molecules and CAS numbers are given. In addition, a table with optimum MS/MS and MS^E conditions used for the interpretation of these compounds are also given (Table SI 2). Furthermore two figures, one reporting fragmentation pathway of ketamine and MS^E (HE) spectra of the $[M+H]^+$ ion of both ketamine and its deuterated analogue (Figure SI 1), and another including a fragmentation route of opiates via morphinan backbone (Figure SI 2), are added to have supportive visual information on the written text.

Supporting information associated with this article can be found, in the online version, at http://onlinelibrary.wiley.com/doi/10.1002/jms.1963/suppinfo and in this chapter after section "References".

References

[1] T.A. Ternes. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* **1998**, *32*, 3245.

[2] S.D. Richardson. Environmental mass spectrometry: emerging contaminants and current issues. *Anal. Chem.* **2010**, *82*, 4742.

[3] S. Castiglioni, E. Zuccato, C. Chiabrando, R. Fanelli, R. Bagnati. Mass spectrometric analysis of illicit drugs in wastewater and surface water. *Mass Spectrom. Rev.* **2008**, *27*, 378.

[4] C. Postigo, M.J. Lopez de Alda, D. Barceló. Analysis of drugs of abuse and their human metabolites in water by LC-MS^2: A non-intrusive tool for drug abuse estimation at the community level. *Trends Anal. Chem.* **2008**, *27*, 1053.

[5] F. Hernández, O.J. Pozo, J.V. Sancho, F.J. Lopez, J.M. Marín, M. Ibañez. Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS^2 using triple quadrupole and hybrid quadrupole time-of-flight analyzers. *Trends Anal. Chem.* **2005**, *24*, 596.

[6] M. Krauss, H. Singer, J. Hollender. LC-high resolution MS in environmental analysis: from target screening to the identification of unknowns. *Anal. Bioanal. Chem.* **2010**, *397*, 943.

[7] J.A. van Leerdam, A.C. Hogenboom, M.M.E. van der Kooi, P. de Voogt. Determination of polar 1H-benzotriazoles and benzothiazoles in water by solid-phase extraction and liquid chromatography LTQ FT Orbitrap mass spectrometry. *Int. J. Mass Spectrom.* **2009**, *282*, 99.

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

[9] R.J.B. Peters, A.A.M. Stolker, J.G.J. Mol, A. Lommen, E. Lyris, Y. Angelis, A. Vonaparti, M. Stamou, C. Georgakopoulus, M.W.F. Nielen. Screening in veterinary

drug analysis and sports doping control based on full-scan accurate mass spectrometry. *Trends Anal. Chem.* **2010**, *29*, 1250.

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

[11] A.C. Hogenboom, J.A. van Leerdam, P. de Voogt. Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography - hybrid linear ion trap Orbitrap mass spectrometry. *J. Chromatogr. A* **2009**, *1216*, 510.

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

[13] E. Zuccato, C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea, R. Fanelli. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. *Environ. Health* **2005**, *4:14*, http://www.ehjournal.net/content/4/1/14.

[14] A.L.N. van Nuijs, B. Pecceu, L. Theunis, N. Dubois, C. Charlier, P.G. Jorens, L. Bervoets, R. Blust, H. Meulemans, H. Neels, A. Covaci. Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium. *Addiction*, **2009**, *104*, 734.

[15] A.L.N. van Nuijs, S. Castiglioni, I. Tarcomnicu, C. Postigo, M. Lopez de Alba, H. Neels, E. Zuccato, D. Barcelo, A. Covaci. Illicit drug consumption estimations derived from wastewater analysis: a critical review. *Sci. Total Environ.* doi: 10.1016/j.scitotenv.2010.05.030.

[16] T. Belal, T. Awad, J. DeRuiter, C. Randall Clark. GC-MS studies on acylated derivatives of 3-methoxy-4-methyl- and 4-methoxy-3-methyl-phenethylamines: Regioisomers related to 3,4-MDMA. *Forensic Sci. Int.* **2008**, *178*, 61.

[17] M. Pieri, L. Castiglia, N. Miraglia, R. Guadagni, L. Malorni, N. Sannolo, A. Acampora, E. Della Casa. Study of the fragmentation pattern of ketamine-heptafluorobutyramide by gas chromatography/electron ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 49.

[18] B. Maralikova, W. Weinmann. Simultaneous determination of Δ9-tetrahydrocannabinol, 11-hydroxy-Δ9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in human plasma by high-performance liquid chromatography/tandem mass spectrometry. *J. Mass Spectrom.* **2004**, *39*, 526.

[19] K.-C. Wang, T.-S. Shih, S.-G. Cheng. Use of SPE and LC/TIS/MS/MS for rapid detection and quantitation of ketamine and its metabolite, norketamine, in urine. *Forensic Sci. Int.* **2005**, *147*, 81.

[20] P.-P. Wang, M.G. Bartlett. Collision-induced dissociation mass spectra of cocaine, and its metabolites and pyrolysis products. *J. Mass Spectrom.* **1998**, *33*, 961.

[21] P.M. Jeanville, E.S. Estapé, I. Torres-Negrón de Jeanville. The effect of liquid chromatography eluents and additives on the positive ion responses of cocaine, benzoylecgonine, and ecgonine methyl ester using electrospray ionization. *Int. J. Mass Spectrom.* **2003**, *227*, 247.

[22] W.F. Smyth. Electrospray ionisation mass spectrometric behaviour of selected drugs and their metabolites. *Anal. Chim. Acta* **2003**, *492*, 1.

[23] K. Riath, R. Neubert, C. Poeaknapo, C. Boettcher, M.H. Zenk, J. Schmidt. Electrospray tandem mass spectrometric investigations of morphinans. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 1262.

[24] C. Poeaknapo, U. Fisinger, M.H. Zenk, J. Schmidt. Evaluation of the mass spectrometric fragmentation of codeine and morphine after 13C-isotope biosynthetic labeling. *Phytochemistry* **2004**, *65*, 1413.

[25] Z. Zhang, B. Yan, K. Liu, T. Bo, Y. Liao, H. Liu. Fragmentation pathways of heroin-related alkaloids revealed by ion trap and quadrupole time-of-flight tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 2851.

[26] S.R. Vande Casteele, M-P. L. Bouche, J.F. Van Bocxlaer. LC-MS7MS in the elucidation of an isomer of the recreational drug methylenedioxy-ethylamphetamine: Mtheylenedioxydimethylamphetamine. *J. Sep. Sci.* **2005**, *28*, 1729.

[27] K. Clauwaert, S. Vande Casteele, B. Sinnaeve, D. Deforce, W. Lambert, C. Van Peteghem, J. Van Bocxlaer. Exact mass measurement of product ions for the structural confirmation and identification of unknown compounds using quadrupole time-of-flight spectrometer: a simplified approach using combined tandem mass spectrometric functions. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1443.

[28] A.C. Hogenboom, W.M.A. Niessen, D. Little, U.A.Th. Brinkman. Accurate mass determination for the confirmation and identification of organic microcontaminants in surface water using on-line solid-phase extraction liquid chromatography electrospray orthogonal-acceleration time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 125.

[29] J.V. Sancho, O.J. Pozo, M. Ibañez, F. Hernández. Potential of liquid chromatography/time-of-flight mass spectrometry for the determination of pesticides and transformation products in water. *Anal. Bioanal. Chem.* **2006**, *386*, 987.

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

[31] P.J. Weaver, A. Laures, J-C. Wolff. Investigation of the advanced functionalities of a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2415.

[32] P.R. Tiller, S. Yu, J. Castro-Perez, K.L. Fillgrove, T.A. Baillie. High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line' approach for metabolite identification studies. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 1053.

[33] W. Weinmann, S. Vogt, R. Goerke, C. Müller, A. Bromberger. Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS. *Forensic Sci. Int.* **2000**, *113*, 381.

[34] W. Weinmann, M. Goerner, S. Vogt, R. Goerke, S. Pollak. Fast confirmation of 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) in urine by LC/MS/MS using negative atmospheric-pressure chemical ionisation (APCI). *Forensic Sci. Int.* **2001**, *121*, 103.

[35] G. D'Ascenzo, A. Di Corcia, A. Gentili, R. Mancini, R. Mastropasqua, M. Nazzari, R. Samperi. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. *Sci. Total Environ.* **2003**, *302*, 199.

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

[37] C. Postigo, M.J. Lopez de Alda, D. Barceló. Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography-electrospray-tandem mass spectrometry. *Anal. Chem.* **2008**, *80*, 3123.

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

[39] M.R. Boleda, M.T. Galceran, F. Ventura. Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2007**, *1175*, 38.

[40] F. Hernández, J.V. Sancho, L. Bijlsma. Wide-scope screening of illicit drugs in urban wastewater by UHPLC-QTOF MS. In: *Illicit drugs in the environment: occurrence, analysis, and fate using mass spectrometry,* (Eds: S. Castiglioni, E. Zuccato, R. Fanelli), *John Wiley, New York, ISBN: 978-0-470-52954-6,* ***2011****,* 69.

[41] M. Huerta-Fontela, M.T. Galceran, J. Martin-Alonso, F. Ventura. Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain. *Sci. Tot. Env.* **2008**, *397*, 31.

[42] United Nations Office of Drug and Crime; World Drug Report **2009**, http://www.unodc.org/documents/wdr/WDR_2009/WDR2009_eng_web.pdf.

[43] T.L. Jones-Lepp, D.A. Alvarez, J.D. Petty, J.N. Huckins. Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents. *Arch. Environ. Contam. Toxicol.* **2004**, *47*, 427.

[44] C. Postigo, M.J. Lopez de Alda, D. Barceló. Drugs of abuse and their metabolites in the Ebro River basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation. *Environ. Int.* **2010**, *36*, 75.

[45] E. Zuccato, S. Castiglioni, R. Bagnati, C. Chiabrando, P. Grassi, R. Fanelli. Illicit drugs a novel group of environmental contaminants. *Water Res.* **2008**, *42*, 961.

[46] T.H. Boles, M.J.M. Wells. Analysis of amphetamine and methamphetamine as emerging pollutants in wastewater and wastewater-impacted streams. *J. Chromatogr. A* **2010**, *1217*, 2561.

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

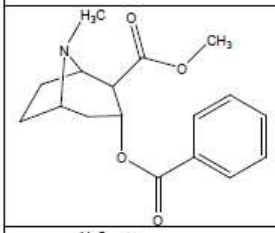
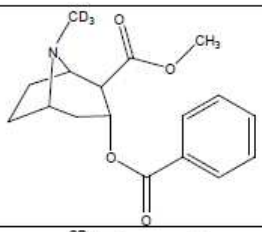
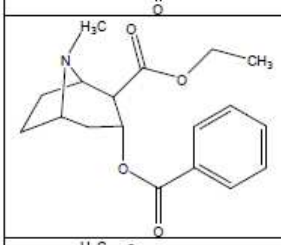
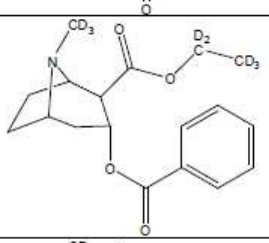
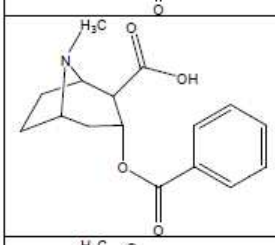
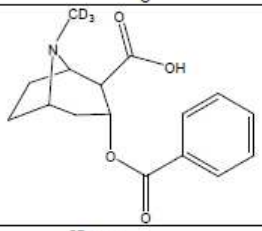
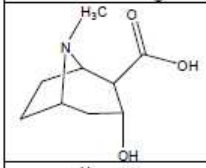
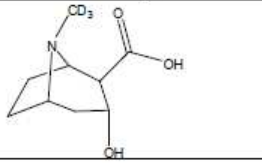
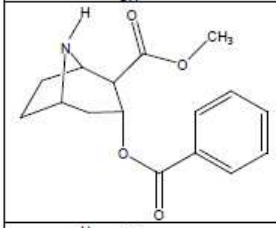
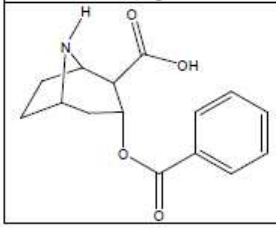
[48] O.J. Pozo, J.V. Sancho, M. Ibáñez, F. Hernández, W.M.A. Niessen. Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls. *Trends Anal. Chem.* **2006**, *25*, 1030.

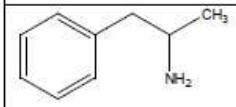
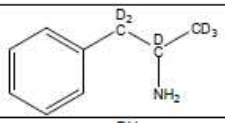
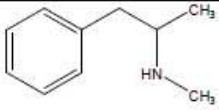
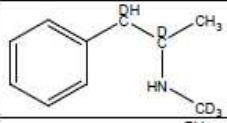
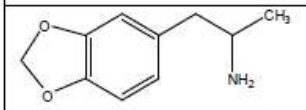
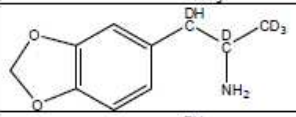
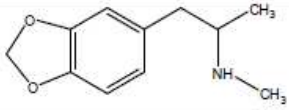
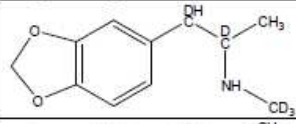
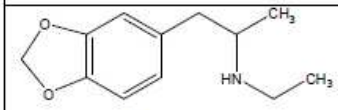
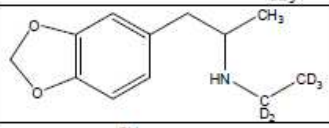
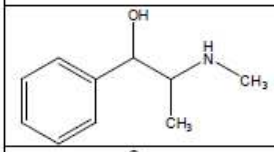
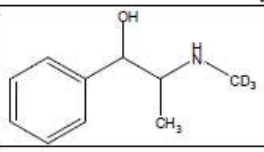
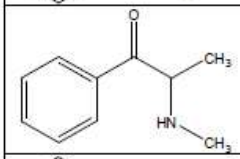
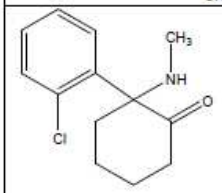
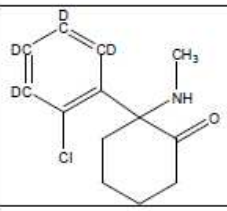
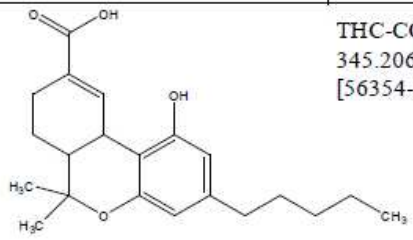
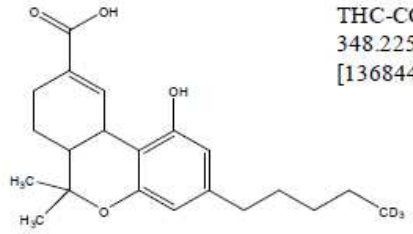
[49] European Union Decision 2002/657/EC 17 August 2002. *Off. J. Eur. Commun.* **2002**, *8*, L221.

[50] T. Portolés, E. Pitarch, F.J. López, F. Hernández. Development and validation of a rapid and wide-scope qualitative screening for detection of organic pollutants in natural water and wastewater by gas chromatography-time of flight mass spectrometry. *J. Chromatogr. A* **2011**, *1218 (2)*, 303.

Supporting information

Table SI 1: Chemical structure, exact mass of the protonated molecule, and CAS number of the selected illicit drugs and their deuterated analogues.

Structure	Compound Exact mass $[M+H]^+$ CAS number	Structure	Compound Exact mass $[M+H]^+$ CAS number
	Cocaine 304.1549 [50-36-2]		Cocaine-d_3 307.1737 [65266-73-1]
	Cocaethylene 318.1705 [529-38-4]		Cocaethylene-d_8 326.2207 [152521-09-0]
	Benzoylecgonine 290.1392 [519-09-5]		Benzoylecgonine-d_3 293.1580 [115732-68-8]
	Ecgonine 186.1130 [5796-31-6]		Ecgonine-d_3 189.1318 [N/A]
	Norcocaine 290.1392 [61585-22-6]		
	Norbenzoylecgonine 276.1236 [60426-41-7]		

Structure	Compound Exact mass $[M+H]^+$ CAS number	Structure	Compound Exact mass $[M+H]^+$ CAS number
	Amphetamine 136.1126 [300-62-9]		Amphetamine-d_6 142.1503 [N/A]
	Methamphetamine 150.1282 [537-46-2]		Methamphetamine-d_5 155.1596 [60124-88-1]
	MDA 180.1024 [4764-17-4]		MDA-d_5 185.1338 [136765-42-9]
	MDMA 194.1181 [42542-10-9]		MDMA-d_5 199.1495 [13675-43-0]
	MDEA 208.1337 [82801-81-8]		MDEA-d_5 213.1651 [160227-43-0]
	Ephedrine 166.1232 [50-98-6]		Ephedrine-d_3 169.1420 [N/A]
	Methcathinone 164.1075 [152610-69-0]		
	Ketamine 238.0998 [1867-66-9]		Ketamine-d_4 242.1249 [N/A]
	THC-COOH 345.2066 [56354-06-4]		THC-COOH-d_3 348.2254 [136844-96-7]

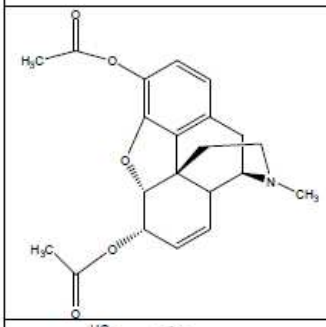
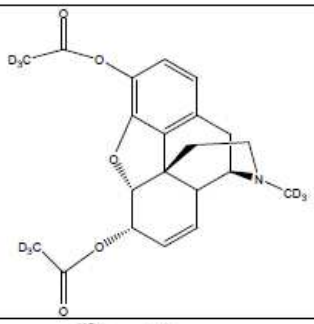
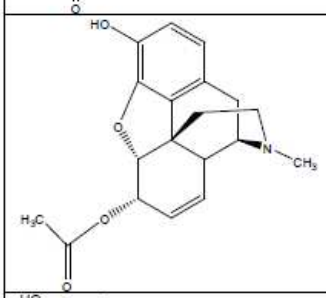
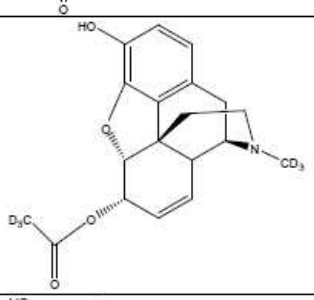
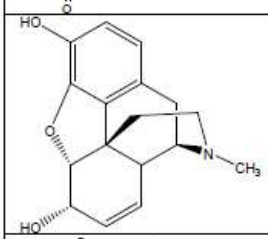
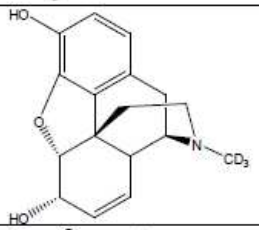
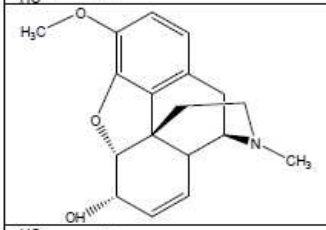
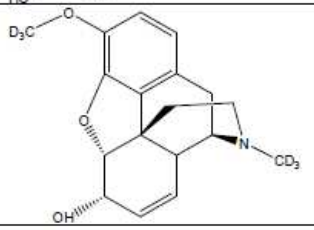
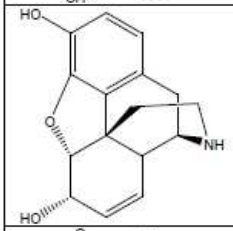
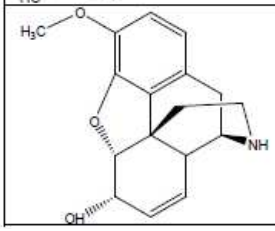
Structure	Compound Exact mass $[M+H]^+$ CAS number	Structure	Compound Exact mass $[M+H]^+$ CAS number
	Heroin 370.1654 [561-27-3]		Heroin-d_9 379.2219 [N/A]
	6-MAM 328.1549 [2784-73-8]		6-MAM-d_6 334.1925 [152477-90-2]
	Morphine 286.1443 [57-27-2]		Morphine-d_3 289.1631 [67293-88-3]
	Codeine 300.1599 [76-57-3]		Codeine-d_6 306.1976 [N/A]
	Normorphine 272.1286 [466-97-7]		
	Norcodeine 286.1443 [467-15-2]		

Table SI 2: Optimum MS/MS [(a)] and MS^E [(b)] conditions used for the interpretation of spectra of illicit drugs and their metabolites. (ESI operated in positive ionization mode).

Compound	Retention time (min)	Cone voltage (V)	High Collision Energy (eV)
THC-COOH	8.77	25	10-45
THC-COOH-d3	8.77	25	10-40
Ketamine	3.76	15	10-40
Ketamine-d4	3.74	10	10-40
Amphetamine	3.05	10	10-50
Amphetamine-d6	3.03	10	10-40
MDA	3.08	10	10-45
MDA-d5	3.03	10	10-40
MDEA	3.43	15	10-40
MDEA-d5	3.42	15	10-45
MDMA	3.12	10	10-40
MDMA-d5	3.11	10	10-45
Methamphetamine	3.15	15	10-40
Methamphetamine-d5	3.12	15	10-45
Benzoylecgonine	3.78	15	10-40
Benzoylecgonine-d3	3.78	15	10-40
Cocaethylene	4.67	20	10-40
Cocaethylene-d8	4.66	20	10-40
Cocaine	4.10	15	10-40
Cocaine-d3	4.10	20	10-40
Ecgonine	1.00	20	10-50
Ecgonine-d3	0.99	20	10-45
Norbenzoylecgonine	3.92	15	10-45
Norcocaine	4.37	15	10-40
6-Acetylmorphine	2.83	25	20-60
6-Acetylmorphine-d6	2.79	25	20-50
Codeine	2.45	20	20-60
Codeine-d6	2.46	25	20-50
Ephedrine	2.66	10	10-45
Ephedrine-d3	2.64	10	10-40
Heroin	3.97	30	20-60
Heroin-d9	3.95	35	20-50
Methcathinone	2.64	15	10-40
Morphine	1.64	30	20-50
Morphine-d3	1.62	35	20-50
Norcodeine	2.55	25	20-50
Normorphine	1.54	25	20-50

a) MS/MS conditions, selecting the protonated molecule $[M+H]^+$ as precursor ion.

b) Conditions used for MS^E in the HE mode. The collision energy applied in the LE mode was in all cases 4eV.

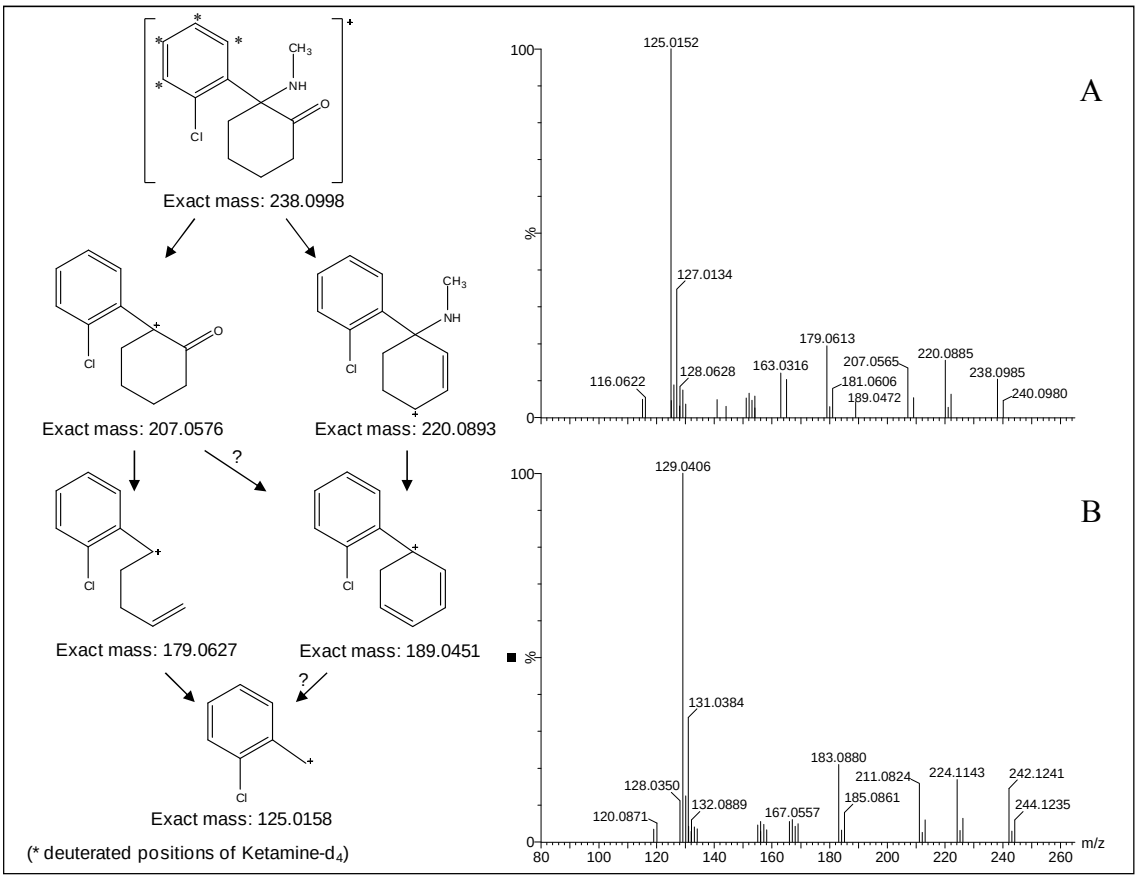


Figure SI 1. Proposed ketamine fragmentation pathway (left). MS^E (HE) spectra (right) of $[M+H]^+$ of ketamine (A), ketamine-d_4 (B).

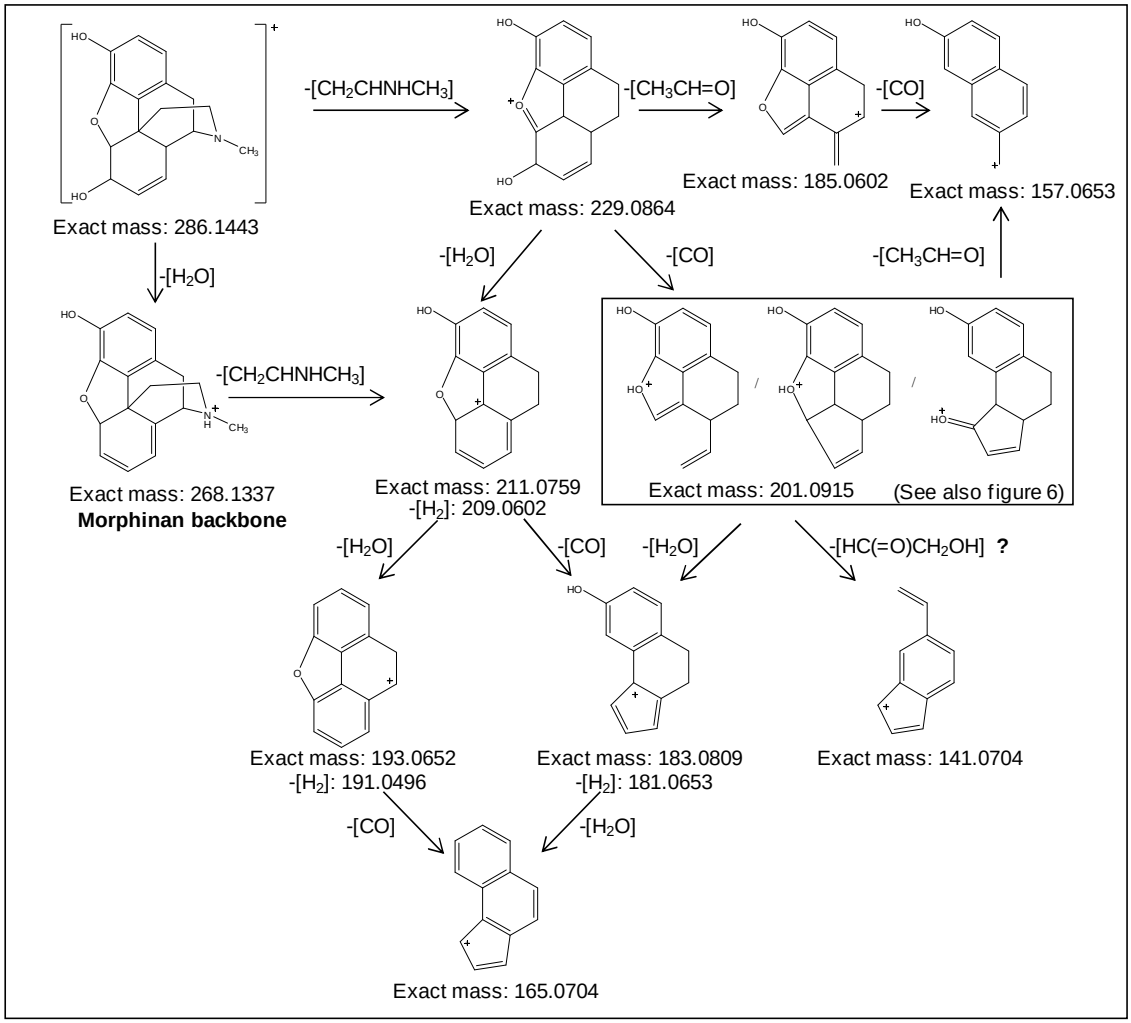


Figure SI 2. Proposed fragmentation route of opiates via morphinan backbone.

Chapter 4.2.2, scientific article 7

Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment

Lubertus Bijlsma, Clara Boix, Wilfried M.A. Niessen, María Ibáñez, Juan V. Sancho, Félix Hernández

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Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment

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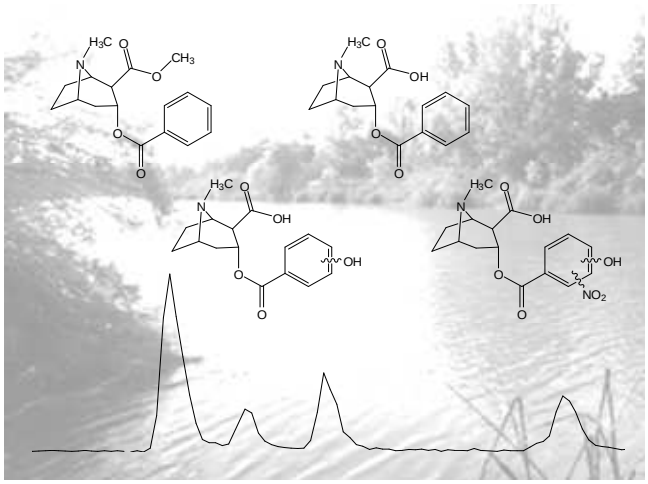
Graphical Abstract



Highlights

- Cocaine and benzoylecgonine degradation/transformation products investigated in water
- Hydrolysis, chlorination and photo-degradation studied under laboratory conditions
- Several TPs discovered and tentatively elucidated by high resolution MS
- Structures of non-previously reported TPs have been suggested
- Several reported/known TPs but also new TPs were found in sewage and surface water

Abstract

In this work, ultra-high-performance liquid chromatography (UHPLC) coupled to a hybrid quadrupole time-of-flight mass spectrometer (QTOF MS) has allowed the discovery and elucidation of degradation products of cocaine and its main metabolite benzoylecgonine (BE) in water. Spiked surface water was subjected to hydrolysis, chlorination and photo-degradation (both ultraviolet irradiation and simulated sunlight). After degradation of cocaine, up to sixteen compounds were detected and tentatively identified (1 resulting from hydrolysis; 8 from chlorination; 7 from photo-degradation), three of which are well known cocaine metabolites (BE, norbenzoylecgonine and norcocaine). Regarding BE degradation, up to ten compounds were found (3 from chlorination; 7 from photo-degradation), including one known metabolite (norbenzoylecgonine). Since reference standards were available for the major metabolites, they could be confirmed using information on retention time and fragment ions. The other degradates resulted from chlorination, dealkylation, hydroxylation and nitration, or from a combination of these processes. Several influent and effluent sewage water, and surface water samples were then screened for the identified compounds (known and unknown) using UHPLC–tandem MS with triple quadrupole. BE, norcocaine and norbenzoylecgonine were identified in these samples as major metabolites. Four previously unreported degradates were also found in some of the samples under study, illustrating the usefulness and applicability of the degradation experiments performed in this work.

Keywords

Cocaine; Degradation and transformation products; Water; Time-of-flight mass spectrometry

1. Introduction

Cocaine use has increased during the last decade and is the illicit drug with the second-highest consumption in Europe, behind only cannabis (EMCDDA, 2010). After consumption and excretion, cocaine enters the sewage treatment plants (STPs) as the parent drug or as human metabolites (mainly benzoylecgonine (BE)) and may end up in the receiving surface waters as a consequence of incomplete elimination in the STPs. In most studies, if the presence of cocaine in the aquatic environment is reported, only the parent compound and a few relevant metabolites, commonly BE and cocaethylene or ecgonine methyl ester are included (Baker and Kasprzyk-Hordern, 2011). Occasionally, in monitoring studies dealing with sewage- and surface water, some minor metabolites have been found, such as norBE and norcocaine (e.g. Chiaia et al., 2008; Zuccato et al., 2008; Bijlsma et al., 2009 and Bisceglia et al., 2010). Although concentrations reported in surface water are generally low (i.e. 7–60 ng/L for cocaine and 15–191 ng/L for BE (Huerta-Fontela et al., 2008 and Gheorghe et al., 2008)), there is a potential negative impact of their presence in the aquatic ecosystem (Binelli et al., 2012). Especially, the effects of combined exposure to multiple compounds are of potential concern.

In order to evaluate the hazard in the water cycle, not only removal of the parent compounds and metabolites in the treatment processes must be taken into account, but also the possible formation of degradation/transformation products (TPs). In some countries (e.g. Italy), chlorination is progressively abandoned because of its potential for generating unwanted TPs and replaced by UV irradiation (Antonelli et al., 2008). Furthermore, after incomplete elimination during chlorination (Huerta-Fontela et al., 2008 and Boleda et al., 2011), cocaine and BE which ended up in surface water may be exposed to natural sunlight and produce photo-degradation products. The same would occur for cocaine and BE still present in treated wastewater when no tertiary treatment is applied in the STP (e.g. Gheorghe et al., 2008; Huerta-Fontela et al., 2008; Bijlsma et al., 2009 and Bisceglia et al., 2010). Despite the fact that some TPs are more persistent or might exhibit similar toxicity than their parent compounds (Farré et al., 2008; Kern et al., 2009; Fatta-Kassinos et al., 2011 and Metz et al., 2011), the research on TPs of

illicit drugs has received little attention. Nevertheless, investigation of TPs is of importance to know the overall contribution of chemicals in the environment. Information on potential TPs that may be present in the environment can be used to set-up monitoring studies in order to get a wider and more realistic view on the impact of cocaine on the aquatic environment.

The identification of TPs in the aquatic environment, especially unknown ones, is a challenging task for analytical chemists and commonly various techniques and/or analytical reference standards are necessary for a reliable confirmation (Wick et al., 2011). An important analytical tool in the elucidation of TPs is high resolution mass spectrometry (HRMS), with analyzers like Orbitrap and time-of-flight (TOF). The accurate mass full-spectrum acquisition and the possibility to obtain fragment ions by coupling HRMS to ion trap or quadrupole analyzers is highly suitable and helpful for the proposal of convincing molecular structures (Ibañez et al., 2004; Farré et al., 2008; Quintana et al., 2010 and Metz et al., 2011).

Laboratory degradation experiments in combination with HRMS are one of the most useful tools to identify TPs that can be formed in the aquatic environment. They have been applied mainly to elucidate pesticide and pharmaceutical TPs formed in water (Ibañez et al., 2004; Hernández et al., 2008; Quintana et al., 2010 and Wick et al., 2011). Treatment conditions applied by STPs, e.g. chlorination and UV irradiation, can be simulated, as well as natural sunlight. The most important TPs identified can subsequently be included in multi-residue LC tandem MS methods with triple quadrupole. This has allowed the detection of parent compounds and of their related TPs in sewage-, surface- and/or drinking water (Hernández et al., 2008; Quintana et al., 2010 and Wick et al., 2011), and illustrates the importance of investigating TPs.

The use of MS^E is an attractive option, which is feasible working with hybrid QTOF MS instruments. Using this approach, information on both (de)protonated molecules and their fragment ions is acquired simultaneously in a single injection (Hernández et al., 2011). The accurate mass measurement of the (de)protonated molecule generally allows the assignment of a highly probable molecular formula. Subsequently, fragment

ions as well as neutral losses can be investigated in order to elucidate the structure of the TPs detected. Available software for the detection of metabolites and TPs is usually offered by MS manufacturers. They compare and contrast data of a presumptive positive sample with a control or blank sample. This facilitates data processing and might even detect (low abundant) compounds overlooked by visual inspection.

The objective in this paper was to perform a study on TPs of cocaine and BE that might be found in the aquatic environment. Several laboratory controlled degradation experiments (i.e. hydrolysis, chlorination, and photo-degradation under ultraviolet (UV) irradiation and simulated sunlight) have been carried out and the TPs formed investigated by LC–QTOF under MS^E mode. To the best of our knowledge, several unknown TPs reported in this study have not previously described in the literature. In a subsequent step, influent and effluent sewage water, and also surface waters, were searched for the identified TPs.

2. Materials and methods

2.1. Reagents and chemicals

Cocaine, norcocaine, BE and norbenzoylecgonine (norBE) reference standards were purchased from the National Measurement Institute (Pymble, Australia) and Cerilliant (Round Rock, TX, USA). Standard solutions of cocaine and BE were prepared at 500 mg/L in acetonitrile (ACN) and methanol (MeOH), respectively. Intermediate work solutions (50 mg/L) were made by diluting the solution ten times with MeOH.

HPLC-grade MeOH, ACN and formic acid (FA) were acquired from Scharlau (Barcelona, Spain). Sodium hypochlorite solution (available chlorine 10%) was obtained from Sigma-Aldrich. A Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA) was used to obtain the HPLC grade water. Leucine enkephalin and imazalil were purchased from Sigma-Aldrich and Dr. Ehrenstorfer (Augsburg, Germany), respectively.

Solid-phase extraction (SPE) cartridges (Oasis-HLB; 3 mL, 60 mg) were purchased from Waters (Milford, MA, USA). Prior to use, the SPE cartridges were conditioned by washing and rinsing with 3 mL of MeOH and 3 mL of Milli-Q water.

2.2. Degradation experiments

Blank surface water from the Mijares River (Castellón, Spain) was collected in November 2010 and used for all laboratory controlled experiments. Surface water (pH 8.1) was selected in order to simulate reality, as it contains matrix components which may affect degradation.

Surface water used for hydrolysis, chlorination and photo-degradation experiments was spiked with cocaine or BE at a concentration of 0.5 mg/L. This relatively high concentration allowed better evaluation of degradation products, and especially facilitated the detection of minor TPs. Non-spiked surface water samples were subjected to the same degradation processes and used as control samples.

The hydrolysis and chlorination experiments were performed at room temperature and in darkness. Regarding chlorination, 40 µL of ten-fold diluted sodium hypochlorite solution was added to 50 mL of each surface water sample. During the experiment, 2 mL aliquots of the water sample were collected at several time intervals (0, 30 min, 1, 3, 10 h, 1, 3, 7, 11 and 15 days for hydrolysis; and 0, 30 min, 1 and 3 h for chlorination), after stirring of the water solutions, and were immediately stored at − 20 °C until analysis.

Photo-degradation experiments were carried out under UV irradiation and simulated sunlight. UV irradiation was performed using a mercury lamp with its main output at 254 nm. 250 mL surface water samples were kept in quartz glass vessels at a distance of ~ 15 cm from the lamp. The experiment was carried out in a fume hood at room temperature over a period of 72 h under constant stirring of the samples. Sunlight was simulated using a solar simulation system (Suntest XLS+, Atlas MTT, Linsengericht, Germany), equipped with a xenon arc lamp as radiation source and a solar light filter allowing a wavelength in the range of 300–800 nm. The radiation intensity was set to 500 W/m^2 and the light dose per hour of irradiation to 1.8 MJ/h. In this way, 90 irradiation hours corresponds to 15 days of natural sun light (dose: 288 MJ/m^2). The degradation was performed using 250 mL closed quartz glass vessels and sample temperature was set to 25 °C in order to minimize sample evaporation and possible thermal transformation. Aliquots were sampled after stirring of the water solution. The first 2 mL

water aliquots were analyzed, prior to the irradiation experiments (t = 0). During irradiation experiments, 2 mL water samples were taken at different time intervals (see hydrolysis experiment), and immediately stored at − 20 °C until analysis.

2.3. Instrumentation

For identification and elucidation of TPs, a Waters Acquity ultra-high-performance liquid chromatography (UHPLC) system was interfaced to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Premier, Waters Micromass) equipped with an orthogonal Z-spray electrospray ionization interface (ESI) operating in both positive-ion and negative-ion mode and controlled by MassLynx v 4.1 software. Leucine enkephalin was used as the lock mass (*m/z* 556.2771 in positive-ion and *m/z* 554.2615 in negative-ion mode) ensuring typically mass errors below 2 mDa.

The UHPLC separation was performed using an Acquity UPLC BEH C18, 1.7 μm particle size analytical column, 100 mm × 2.1 mm (Waters). The mobile phases used were A = H_2O and B = MeOH, both with 0.01% FA. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10%; 9 min, 90%; 11 min, 90%; 11.1 min, 10%; 14 min, 10%. The flow rate was 0.3 mL/min.

For MS^E experiments, two acquisition functions were created and simultaneously used within the same run: the low-energy function (LE) with a collision energy of 4 eV, where mainly the (de)protonated intact molecules are observed, and the high energy (HE) function with a collision energy ramp ranging from 15 to 40 eV, where fragmentation is promoted. The same collision energy ramp was used for additional MS/MS experiments. The optimized cone voltage (15 V) and collision energy ramp were identical for both cocaine and BE and seemed therefore most adequate for the screening of their corresponding degradation products. Further details on instrument operating conditions can be found elsewhere (Hernández et al., 2011).

For screening of TPs in sewage waters, a TQD triple quadrupole (QqQ) mass spectrometer with electrospray ionization source (Waters) was used. Chromatographic separation was performed using the same analytical column and gradient as used in

UHPLC–QTOF analysis. The analysis of surface waters was performed under similar conditions using the TQS (QqQ) mass spectrometer (Waters).

2.4. Elucidation/identifaction procedure

Waters MetaboLynx software (an application manager within MassLynx) was used to compare accurate mass data of spiked and blank (non-spiked) samples from the laboratory experiments.

The data comparison by MetaboLynx was performed in two ways. First, for expected TPs, (bio)transformation processes reported in the literature were included in the processing settings. These consisted of a mass window ± 10 mDa for extracted ion chromatograms (XICs) of each specific exact mass; peaks with less than 10 area units were eliminated. Second, searching for unexpected TPs was performed by mass spectral comparison of non-spiked versus spiked samples. XICs were automatically generated for each sample (spiked and non-spiked) over a range from *m/z* 70 to 550 Da, at 1 Da mass window, and compared.

The most likely elemental compositions of (de)protonated molecules were calculated based on accurate mass LE spectra of the peaks of interest. The accurate mass HE spectra were then used to calculate possible elemental compositions of fragment ions. Assuming that most TPs share similar fragmentation pathways with the parent drug (Wang and Bartlett, 1998 and Bijlsma et al., 2011), fragmentation was compared to that of cocaine and BE, and the TP structures were proposed.

2.5. Water samples

Five influent and five effluent sewage water samples (24-hour composite) and five surface water grab samples from different locations of the Comunidad Valenciana (Eastern Spain) were collected and immediately stored at − 20 °C. Sewage water was collected from STPs of Castellón and Benicàssim, while surface water was collected from the Albufera national park of Valencia.

100 mL of five-fold diluted (with MilliQ) influent wastewater, 100 mL of effluent wastewater or 100 mL surface water was taken for analysis. The samples were loaded

onto the HLB cartridges by gravity, and then cartridges were vacuum-dried for 10 min. Analytes were eluted with 5 mL of MeOH. The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen and reconstructed in 1 mL of 10:90 MeOH:H_2O. Analyses of cocaine and BE TPs were performed by injecting 20 μL of the final extract into the UHPLC–TQD system (sewage water) or 100 μL in the UHPLC–TQS system (surface water).

3. Results and discussion

Many known TPs of environmental contaminants share similar fragmentation pathways as their parent molecules. Then, knowledge of structures of fragment ions and basic fragmentation rules are helpful for achieving confident TP structure proposals. Isotope fit, Double Bound Equivalent (DBE), and accurate mass of fragments observed in the HE function were used to discard potential chemical formulas in order to obtain the most plausible structures of TPs.

The fragmentation of cocaine and BE has been studied previously by our own group (Bijlsma et al., 2011) and by others (Wang and Bartlett, 1998). This has facilitated the elucidation of some of the TPs found in this work. The most abundant fragment ions in the mass spectra of both compounds are *m*/z 105 ($C_6H_5CO^+$) and a fragment due to the neutral loss of benzoic acid (122 Da). Subsequent fragmentation of the resulting ion $[M + H - 122]^+$ can produce fragments with *m*/z 150, 122, 119, 108, 91 and 82, involving a further loss of methanol or water or elimination of part of the bicyclic ring system, followed by hydrogen rearrangement.

Proposed structures for the TPs found in this work are shown in **Fig. 1**.

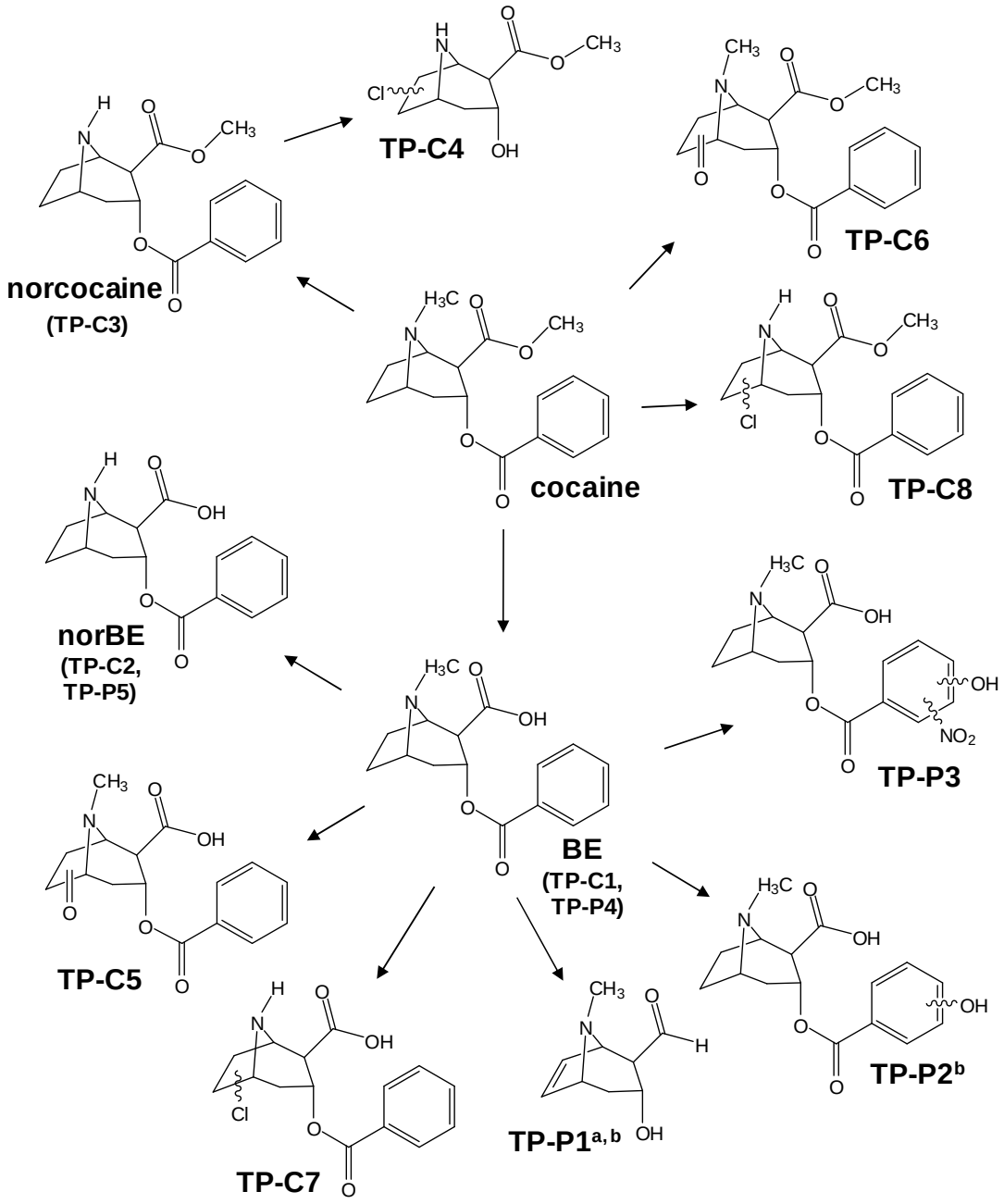


Figure 1. Structure proposals for the identified TPs (TP-Cx were observed after chlorination and TP-P were observed after photo-degradation). [a]TP only observed from photo-degradation of BE. [b]TP detected in sewage and environmental waters.

3.1. Hydrolysis

Gheorghe et al. (2008) performed a detailed study on the stability of BE and cocaine in surface and wastewater, testing at different temperatures and pH values in order to establish optimal conditions for sample storage. Degradation of cocaine was minimal at − 20 °C and pH 2. However, in our study, realistic environmental conditions were chosen for the experiments without any adjustment of pH and temperature. It is therefore likely that besides hydrolysis, potential biodegradation might also occur. To some extent, these processes may yield the same products.

Complete cocaine and some BE degradation was observed in surface water after keeping the solution in darkness at room temperature for 15 days (data not shown). Cocaine was mainly transformed into BE through chemical hydrolysis of cocaine ester bonds. Ecgonine methyl ester (EME), another hydrolytic product reported for cocaine (Postigo et al., 2011), was not observed. EME is presumed to be solely an in vivo metabolite as a result of enzymatic hydrolysis and for that reason it is unlikely to be formed during cocaine degradation in water (Klette et al., 2000). Gheorghe et al. (2008) had similar results to the present work, where cocaine and EME degraded in spiked surface water, while BE initially increased owing to the possible chemical hydrolysis of cocaine.

3.2. Chlorination

Table 1 summarizes the TPs of cocaine and BE formed during chlorination. Retention times and experimental *m/z*-values, proposed elemental composition of the protonated TPs and their fragment ions, the mass error in mDa, and the double bond equivalent (DBE) are given.

Chlorination TPs of cocaine and BE were investigated under the experimental conditions described in Section 2.2. High chlorine concentration (8 mg/L) was used, similar to the conditions employed by STPs for wastewater treatment. In previous studies on acidic pharmaceutical TPs, ascorbic acid was found to be an effective quenching agent to prevent further degradation with chlorine (Quintana et al., 2010).

Table 1. Proposed elemental composition of protonated TPs and their fragments ions obtained during chlorination of cocaine and BE, retention time (min), accurate mass (*m*/z), mass error (mDa) and double bond equivalent (DBE).

Compound (area)[a]	Retention time (min)	Accurate mass (*m*/z)	Chemical formulae	Mass Error (mDa)	DBE
TP-C1[b, c]	3.74	290.1400	$C_{16}H_{20}NO_4$	+0.8	7.5
BE		168.0979	$C_9H_{14}NO_2$	-4.6	3.5
(2059)		150.0896	$C_9H_{12}NO$	-2.3	4.5
		119.0478	C_8H_7O	-1.9	5.5
		105.0325	C_7H_5O	-1.5	5.5
		82.0658	C_5H_8N	+0.1	2.5
TP-C2[b]	3.95	276.1229	$C_{15}H_{18}NO_4$	-0.7	7.5
norBE		154.0853	$C_8H_{12}NO_2$	-1.5	3.5
(680)		136.0744	$C_8H_{10}NO$	-1.8	4.5
		105.0332	C_7H_5O	-0.8	5.5
TP-C3[c]	4.31	290.1391	$C_{16}H_{20}NO_4$	-0.1	7.5
norcocaine		168.0998	$C_9H_{14}NO_2$	-2.7	3.5
(491)		136.0750	$C_8H_{10}NO$	-1.2	4.5
		105.0340	C_7H_5O	0.0	5.5
TP-C4[c]	4.81	220.0729	$C_9H_{15}ClNO_3$	-1.1	2.5
(532)		202.0629	$C_9H_{13}ClNO_2$	-0.6	3.5
		188.0475	$C_8H_{11}ClNO_2$	+0.3	3.5
		120.0210	C_4H_7ClNO	-0.6	1.5
		114.0103	C_5H_5ClN	-0.8	3.5
TP-C5[b]	5.89	304.1193	$C_{16}H_{18}NO_5$	+0.8	8.5
(18)		286.1080	$C_{16}H_{16}NO_4$	+0.1	9.5
		182.0823	$C_9H_{12}NO_3$	+0.6	4.5
		154.0855	$C_8H_{12}NO_2$	-1.3	3.5
		136.0740	$C_8H_{10}NO$	-2.2	4.5
		105.0337	C_7H_5O	-0.3	5.5
TP-C6[c]	6.45	318.1336	$C_{17}H_{20}NO_5$	-0.5	8.5
(270)		286.1061	$C_{16}H_{16}NO_4$	-1.8	9.5
		196.0951	$C_{10}H_{14}NO_3$	-2.3	4.5
		168.1002	$C_9H_{14}NO_2$	-2.3	3.5
		136.0743	$C_8H_{10}NO$	-1.9	4.5
		105.0325	C_7H_5O	-1.5	5.5
TP-C7[b]	7.53	310.0837	$C_{15}H_{17}ClNO_4$	-0.9	7.5
(607)		274.1046	$C_{15}H_{16}NO_4$	-3.3	8.5
		188.0493	$C_8H_{11}ClNO_2$	+1.5	3.5
		170.0352	C_8H_9ClNO	-2.1	4.5
		152.0700	$C_8H_{10}NO_2$	-1.2	4.5
		142.0417	C_7H_9ClN	-0.7	3.5
		134.0592	C_8H_8NO	-1.4	5.5
		105.0329	C_7H_5O	-1.1	5.5

TP-C8[c]	8.27	324.0988	$C_{16}H_{19}ClNO_4$	-1.5	7.5
(3765)		288.1216	$C_{16}H_{18}NO_4$	-2.0	8.5
		202.0598	$C_9H_{13}ClNO_2$	-3.7	3.5
		170.0351	C_8H_9ClNO	-2.2	4.5
		166.0838	$C_9H_{12}NO_2$	-3.0	4.5
		142.0406	C_7H_9ClN	-1.8	3.5
		134.0587	C_8H_8NO	-1.9	5.5
		105.0328	C_7H_5O	-1.2	5.5

[a] Maximum absolute area observed (initial area for parent compounds around 4700)
[b] Also detected in negative ionization mode (-V)
[c] TP only observed from cocaine

However, in the present study, we observed that it affected the stability of some TPs (the monochlorinated TP-C4, -C7 and -C8 were no longer observed after adding ascorbic acid to the sample vials). Therefore, we did not use ascorbic acid addition in our experiments. The sample aliquots taken at different times were frozen, stored and thawed just before analysis. In any case, quenching chlorination seemed not much important in this case, as a fast degradation of cocaine and BE occurred (after 30 min neither cocaine nor BE was observed in sample aliquots analyzed).

The simultaneous acquisition of accurate mass LE and HE spectra and useful isotopic pattern information (distribution of the ^{37}Cl isotope) obtained in the MS^E mode, allowed the detection and tentative identification of several TPs, in a single injection. Among these TPs, some well-known cocaine metabolites, BE (TP-C1), norBE (TP-C2), and norcocaine (TP-C3), were identified and subsequently confirmed by using reference standards. All TPs were determined and identified in the positive-ion mode. Besides the protonated molecules $[M + H]^+$, their sodium adducts $[M + Na]^+$ were also observed surely owing to the presence of sodium in NaClO. Some TPs that contain a carboxylic group could also be analyzed in negative-ion mode; however, analysis under negative mode did not reveal additional TPs to those observed in positive mode.

TP-C4 ($C_9H_{15}ClNO_3^+$, *m*/z 220.0740), with an abundant peak at 4.81 min, may be generated via benzoylester cleavage and chlorination. Initial fragmentation involves losses of water and methanol (to ions with *m*/z 202 and 188, respectively), suggesting that this TP is a secondary product from TP-C3 (norcocaine).

Chlorination of cocaine yielded an intense peak at 8.27 min with $[M + H]^+$ *m/z* 324.1003, named as TP-C8 ($C_{16}H_{19}ClNO_4^+$), corresponding to demethylation of the bridgehead nitrogen and consecutive halogenation (**Fig. S1A**). The fragmentation of this TP was comparable to cocaine and its metabolites, where the most abundant fragments ions are *m/z* 105 ($C_6H_5CO^+$) and *m/z* 202 (loss of benzoic acid, 122 Da) (Wang and Bartlett, 1998). Secondary fragmentation of the ion *m/z* 202 ($[M + H - 122]^+$) involves the loss of either HCl or CH_3OH to ions *m/z* 166 and 170, respectively, the later indicating that initially *N*-demethylation rather than *O*-demethylation occurred. The complete fragmentation pathway for TP-C8 is proposed in **Fig. S1B**. The characteristic chlorine isotopic pattern confirms the presence of Cl in the fragment ions with *m/z* 202, 170 and 142, whereas it is absent in the ions with *m/z* 288, 166, 134 and 105.

Another TP of cocaine, TP-C6 ($[M + H]^+$, *m/z* 318.1336) is has the same nominal mass as cocaethylene ($C_{18}H_{24}NO_4^+$, *m/z* 318.1705), but they could be differentiated both chromatographically and by HRMS, as a difference of 36.9 mDa was observed. The most likely molecular formula for TP-C6 is $C_{17}H_{20}NO_5^+$ (*m/z* 318.1341, Δ 0.5 mDa). Thus, TP-C6 would result from oxidation (+ O–2H) of cocaine during chlorination experiments, which probably occurs on the bicyclic ring system, since the characteristic fragment ion with *m/z* 105 ($C_6H_5CO^+$) is still present.

Chlorination of BE resulted in TP-C5 ($[M + H]^+$, *m/z* 304.1185) and TP-C7 ($C_{15}H_{17}ClNO_4^+$, *m/z* 310.0846) at retention times of 5.89 min and 7.53 min, respectively (**Table 1**). These compounds show similar fragmentation pathways to TP-C6 and TP-C8, respectively, although with an expected mass shift of − 14 in several of the *m/z* values. These TPs were also observed after cocaine chlorination, where BE probably acted as an intermediate.

The most abundant TPs formed after cocaine chlorination corresponded to TP-C8 and TP-C1 (BE), whereas TP-C5 could be considered as minor TP. The abundance of TP-C2 (norBE), solely formed after chlorination of BE, was in the same order of magnitude as TP-C3 (norcocaine), -C4, -C6 and -C7.

The data obtained was not sufficient to predict the exact position of the chlorine or keto group in the unknown TPs (from C4 to C8). The combination of several spectroscopic techniques, such as further analysis by nuclear magnetic resonance (NMR), would be required to definitely elucidate the molecular structure of these compounds. Nevertheless, the information obtained in this study regarding the elemental composition of protonated TPs and their fragment ions will allow screening of these compounds in future monitoring studies. This is of interest to have more realistic and complete information, as these TPs are not included in environmental studies related with the presence of cocaine.

3.3. Photo-degradation

Photo-degradation of cocaine and BE in aqueous solution under simulated sunlight and/or UV irradiation resulted in eight TPs (**Fig. 1**) including two known metabolites: BE (TP-P4) and norBE (TP-P5). The TPs and the data obtained from the QTOF experiments are summarized in **Table 2**. Initially, TP-P2 isomers and TP-P4 were also generated after UV irradiation, but these TPs were effectively removed after 3 and 8 h, respectively.

TP-P1 ($C_9H_{14}NO_2^+$, *m*/z 168.1025), with an abundant peak at 1.93 min, was generated under simulated sunlight of BE. TP-P1 may be produced via cleavage of the benzoyl ester bond, reduction of the carboxylic acid group and dehydrogenation. Its fragmentation under HE acquisition mode involved the loss of water (*m*/z 150) and subsequent loss of formaldehyde (*m*/z 120) and of the bridgehead nitrogen (*m*/z 93). The fact that there is no loss of both H_2O and CO indicates the absence of a carboxylic acid function in this molecule. No confirmative position of the double-bond could be given. However most likely it would be located in the part away from the hydroxyl and aldehyde group.

Table 2. Proposed elemental composition of protonated TPs and their fragments ions obtained during photo-degradation of cocaine and BE, retention time (min), accurate mass (*m*/z), mass error (mDa) and the double bond equivalent (DBE).

Compound (area)[a]	Retention time (min)	Sun/UV[b]	Accurate mass (*m*/z)	Chemical formulae	Mass Error (mDa)	DBE
TP-P1[c]	2.05	Sun	168.1009	$C_9H_{14}NO_2$	-1.6	3.5
(81)			150.0902	$C_9H_{12}NO$	-1.7	4.5
			120.0792	$C_8H_{10}N$	-2.1	4.5
			100.0747	$C_5H_{11}NO$	-1.5	1.5
			93.0686	C_7H_9	-1.8	3.5
TP-P2[d]	2.78	Sun /	306.1332	$C_{16}H_{20}NO_5$	-0.9	7.5
hydroxy-BE	3.07	UV	186.1103[e]	$C_9H_{16}NO_3$	-2.7	2.5
(111)	3.91		168.1014	$C_9H_{14}NO_2$	-1.1	3.5
(46)			150.0903	$C_9H_{12}NO$	-1.6	4.5
(88)			121.0265	$C_7H_5O_2$	-2.5	5.5
			82.0665	C_5H_8N	+0.8	2.5
TP-P3	2.94	Sun	351.1180	$C_{16}H_{19}N_2O_7$	-1.2	8.5
(22)	3.54		168.1004	$C_9H_{14}NO_2$	-2.1	3.5
(39)			166.0118	$C_7H_4NO_4$	-2.2	6.5
			150.0902	$C_9H_{12}NO$	-1.7	4.5
			119.0487	C_8H_7O	-1.0	5.5
			82.0660	C_5H_8N	+0.3	2.5
TP-P4[d]	3.74	Sun /	290.1397	$C_{16}H_{20}NO_4$	+0.5	7.5
BE		UV	272.1304	$C_{16}H_{18}NO_3$	+1.7	8.5
(6189)			168.1047	$C_9H_{14}NO_2$	+2.2	3.5
			150.0946	$C_9H_{12}NO$	+2.7	4.5
			119.0514	C_8H_7O	+1.7	5.5
			105.0357	C_7H_5O	+1.7	5.5
			82.0672	C_5H_8N	+1.5	2.5
TP-P5[d]	3.96	Sun	276.1227	$C_{15}H_{18}NO_4$	-0.9	7.5
norBE			154.0850	$C_8H_{12}NO_2$	-1.8	3.5
(20)			136.0738	$C_8H_{10}NO$	-2.4	4.5
			108.0799	$C_7H_{10}N$	-1.4	3.5
			105.0335	C_7H_5O	-0.5	5.5

[a] Maximum absolute area observed (initial area for parent compounds around 7800)
[b] TP as a result of irradiation under simulated sunlight (Sun) and/or ultraviolet (UV)
[c] TP only observed from BE
[d] Also detected in negative ionization mode (-V)
[e] This fragment ion is not present in the *meta*-hydroxyBE (Tr= 3.07min)

After photo-degradation of both cocaine and BE, three isomeric products named as TP-P2 ($C_{16}H_{20}NO_5^+$, *m*/z 306.1341) were detected at retention times of 2.78, 3.07 and 3.91 min (**Fig. 2A, left**). They would correspond to a hydroxylation product of BE. During photo-degradation of cocaine, BE was formed and readily transformed afterwards acting as a photo-intermediate (**Fig. 3**). In MS^E, the three TP-P2 isomers showed similar fragmentation with fragment ions *m*/z 168 (loss of 138 Da, i.e., benzoic acid + O) and *m*/z 121 ($C_7H_5O_2^+$, corresponding to $C_6H_5CO^+$ + O, *m*/z 105 + 16), indicating that hydroxylation occurs at the phenyl ring. Accordingly, the TP-P2 isomers were presumably *ortho*-, *meta*- and *para*-hydroxy-BE. In vivo, cocaine metabolizes to BE and then to norBE and/or to *meta*-hydroxyl-BE and *para*-hydroxyl-BE (Klette et al., 2000). The suggested hydroxylation products here are similar to the monohydroxylated cocaine products generated by hydrogen peroxide treatment (Tanaka et al., 2002), as a result of solar photo-degradation using a catalyst (titanium dioxide, TiO_2), or by a photo-Fenton reaction (Postigo et al., 2011). In our work, the three isomers seemed to be formed in the photo-degradation experiments as the XIC at the $[M + H]^+$ exact mass (*m*/z 306.1341) revealed. All the three isomers gave fragment ions with *m*/z 168, 150 and 121, whereas only two compounds generated the ion with *m*/z 186 (**Fig. 2A**). This is probably due to the fact that the loss of 120 Da ($C_7H_4O_2$) results in resonance-stabilized neutrals only for the *ortho*- and *para*-analogues (**Fig. 2B**). Thus, the peak at 3.07 min should be the *meta*-hydroxy-BE. Combining this information with literature data on the elution order of *para*- and *meta*-hydroxy-BE (Pichini et al., 2005 and Bisceglia et al., 2010), one can conclude that the isomer at 2.78 min is the *para*-isomer and the one with 3.90 min the *ortho*-isomer.

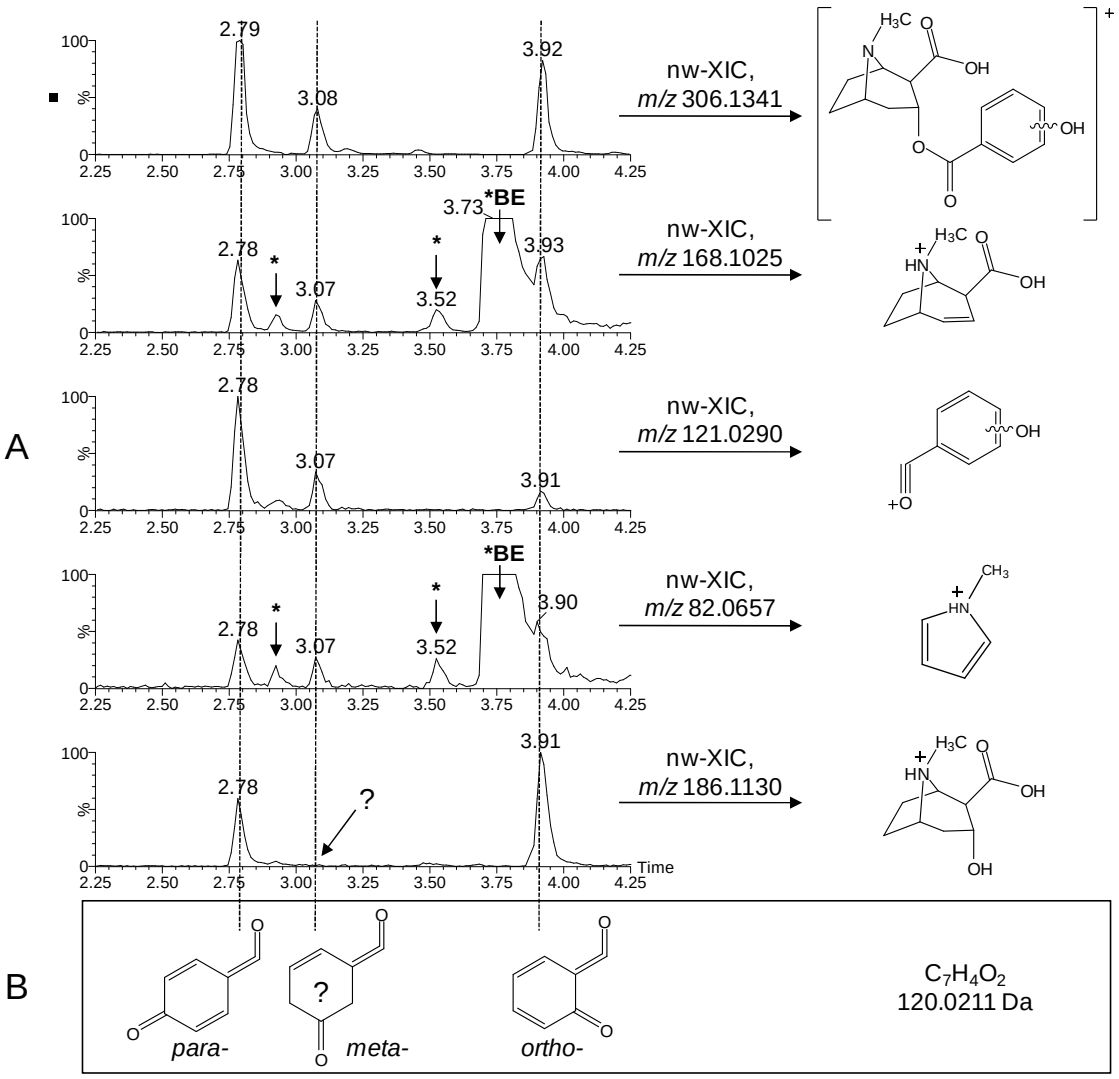


Figure 2. Detection and identification of cocaine and BE photo-degradation TP-P2 by UHPLC-QTOF MS operating under MS^E. (A) narrow-window XICs (± 10 mDa) of TP-P2 and structures suggested for $[M+H]^+$ of TP-P2 and its fragment ions. (B) Structure proposals for the neutral loss of 120 Da ($C_7H_4O_2$). Notice the presence of other chromatographic peaks (marked with *), supporting that other TPs share the same fragmentation.

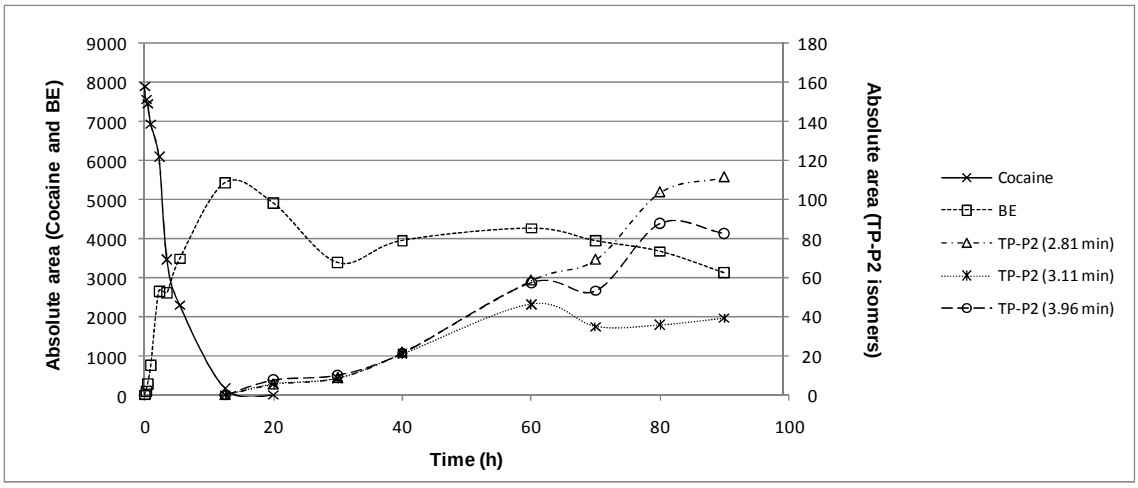


Figure 3. Photo-degradation of cocaine where BE acts as photo-intermediate in the formation of TP-P2.

NorBE (named as TP-P5 in this section) co-eluted with one of the hydroxylated derivatives (Rt = 3.96 and 3.91, respectively) and as a consequence overlapping spectra were obtained in MS^E. In this case, additional product ion MS/MS experiments were performed to obtain "clean" accurate mass spectra of both compounds to confirm their identities.

The accurate mass of two other, less abundant but interesting, unknown isomeric TPs with retention times 2.94 and 3.54 min was determined to be *m*/z 351.1180 (TP-P3) (**Fig. S2**). The most likely molecular formula is $C_{16}H_{18}N_2O_7$ (mass error − 1.2 mDa). Therefore, TP-P3 is suggested to be generated via hydroxylation (+ OH) and nitration (+ NO_2) of BE. The incorporation of a NO_2 group is feasible, since the photo-degradation experiments were carried out using surface water of the Mijares River (Castellón province), where relatively high nitrate concentrations (around 10 mg/L) are normally present owing to the wide use of fertilizers in this agricultural area (Hernández et al., 2008). The presence of the common fragment ions with *m*/z 168, 150, 119 and 82, indicated that hydroxylation and nitration did not take place on the bicyclic ring system, but on the phenyl ring. This could be confirmed by the presence of a major fragment ion with *m*/z 166.0140 ($C_7H_4NO_4^+$) corresponding to $C_6H_5CO^+$ + OH + NO_2 − H_2 (*m*/z 105 + 17 + 46 − 2).

The positions of the NO_2 and OH group could not be definitively determined. From a structural point of view, one might expect more than two chromatographic peaks, since there are various possible combinations regarding the positions of NO_2 and OH. Supposedly, hydroxylation takes place first, because a possible TP corresponding to the nitration of BE ($C_{16}H_{20}NO_4^+ + NO_2$) with *m*/z 335 was not observed, whereas hydroxyl-BE was in fact found, as previously discussed. Based on the effect of a hydroxyl-group on electrophilic substitution, the entrance of NO_2 is probably *ortho*- and *para*-orientated (Morrison and Boyd, 1992). Together with the three possible hydroxy-BE structures, this would result in six conceivable combinations (four *ortho*- and two *para*-orientated). Nevertheless, only two chromatographic peaks were observed at 2.94 and 3.54 min (**Fig. S2**). Possibly, the small differences in polarity allowed co-elution of the *ortho*- and of the *para*-orientated isomers. Owing to interaction via intra-molecular H-bonding of the neighboring − NO_2 and − OH groups in the *ortho*-position the overall polarity of the molecule decreases. As an example, *o*-nitrophenol is retained stronger than *p*-nitrophenol using a reversed-phase analytical column (Masqué et al., 2000). The presence of nitrated derivates indicates influence of the matrix on the degradation of the parent compound.

The use of UHPLC allowed decreasing analysis time with excellent chromatographic resolution. These characteristics are important in terms of sample throughput, separation efficiency and sensitivity (Wilson et al., 2005). In **Fig. 2** and **Fig. S2**, XICs of common fragments show several chromatographic peaks resulting from different TPs. The chromatographic separation of these TPs was important in order to avoid overlapping of spectra acquired using the MS^E approach and to facilitate a reliable identification. Furthermore, the inherent increased sensitivity favored the detection of less abundant TPs.

3.4. Screening of water samples

Screening of cocaine TPs has been performed in several sewage and surface water samples, including the highest number of TPs reported until now. To this aim an UHPLC–MS/MS (QqQ) system was used for the screening of the above suggested TPs in the water samples. This technique is especially suited for target screening in complex

matrices as high sensitivity can be achieved in selected reaction monitoring (SRM) mode. SPE was applied to five influent, five effluent sewage waters and five surface waters in order to pre-concentrate and clean-up the samples (see Section 2.5). Hydrophilic and lipophilic balanced (HLB) cartridges were selected, which demonstrated good efficiency for cocaine, BE and other drugs, pharmaceuticals and metabolites with different physical and chemical characteristics (Gheorghe et al., 2008; Baker and Kasprzyk-Hordern, 2011 and Gracia-Lor et al., 2011). The precursor and product ions, i.e. the MS/MS transitions acquired, were selected (**Table 3**) on the basis of the main ions observed in previous QTOF MS analysis performed along the degradation experiments. A more sensitive QqQ analyzer (i.e. TQS) was used for surface water analysis, due to the lower concentrations expected in comparison with sewage water.

Besides the known metabolites (norcocaine, BE and norBE), TPs of cocaine and BE have been detected, for the first time, in water samples. TP-P1 was found in one influent, four effluents and four surface waters, and TP-P2 isomers were present in four influent and two effluent sewage waters and four surface waters. The TPs had been elucidated after photo-degradation of cocaine and BE by simulated sunlight and/or UV irradiation. Therefore, their presence in influents might be noticed as remarkable, as influent sewage water is normally not exposed to sunlight or UV irradiation. As previously discussed, TP-P2 isomers are suggested to be *ortho*-, *meta*- and *para*-hydroxy-BE. *Meta*-hydroxy-BE and *para*-hydroxy-BE have been reported as in vivo metabolites and might therefore be present in influent sewage water as a consequence of excretion. Nevertheless, *ortho*-hydroxy-BE and TP-P1 were also present in influents. Thus, other processes (e.g. bacterial decomposition) in the sewage system might occur and also play a role in their formation. The presence of TP-P1 and TP-P2 in effluent could be caused by incomplete elimination in the STP or degradation of cocaine and BE during treatment. Furthermore, these TPs were not only found for the first time in influent and effluent sewage waters, but also in surface waters. TPs might enter surface waters by releasing sewage effluents or be formed by photo-degradation via natural sunlight.

Table 3. UHPLC-MS/MS parameters established for the SRM acquisition mode.

Compounds	Retention time (min)	Precursor ion (*m/z*) $[M + H]^+$	CV[a] (V)	CE[b] (eV)	Product ion (m/z)
TP-C1/TP-P4 (BE)	3.74	290.1	40	20	168.2
				30	105.0
				30	82.0
TP-C2/TP-P5 (norBE)	3.95	276.2	45	15	154.1
				20	136.1
				30	105.0
TP-C3 (norcocaine)	4.31	290.1	40	20	168.2
				25	136.1
				30	105.0
TP-C4	4.81	220.1	35	30	188.0
				25	120.0
				30	114.0
TP-C5	5.89	304.1	30	25	154.1
				25	136.0
				30	105.3
TP-C6	6.45	318.1	30	25	286.1
				20	196.2
				25	136.1
TP-C7	7.53	310.1	30	25	188.0
				25	152.0
				35	105.0
TP-C8	8.27	324.1	35	25	288.1
				25	166.1
				25	105.0
TP-P1	2.05	168.1	35	30	150.1
				25	120.1
				30	93.0
TP-P2	2.78	306.1	35	20	168.1
	3.07			15	186.1
	3.91			30	121.0
TP-P3	2.94	351.1	35	35	119.0
	3.54			15	168.1
				35	82.1

[a] CV, cone voltage

[b] CE, collision energy

Fig. 4 shows a positive finding of TP-P1 and TP-P2 isomers in effluent sewage water and in surface water. Although their reference standards were not available, the fact that all the three SRM transitions acquired and that relative retention times to BE (RT_{-TP}/RT_{-BE}) were in good agreement (< 0.01 min) with the TPs identified in degradation experiments give reliability to these findings.

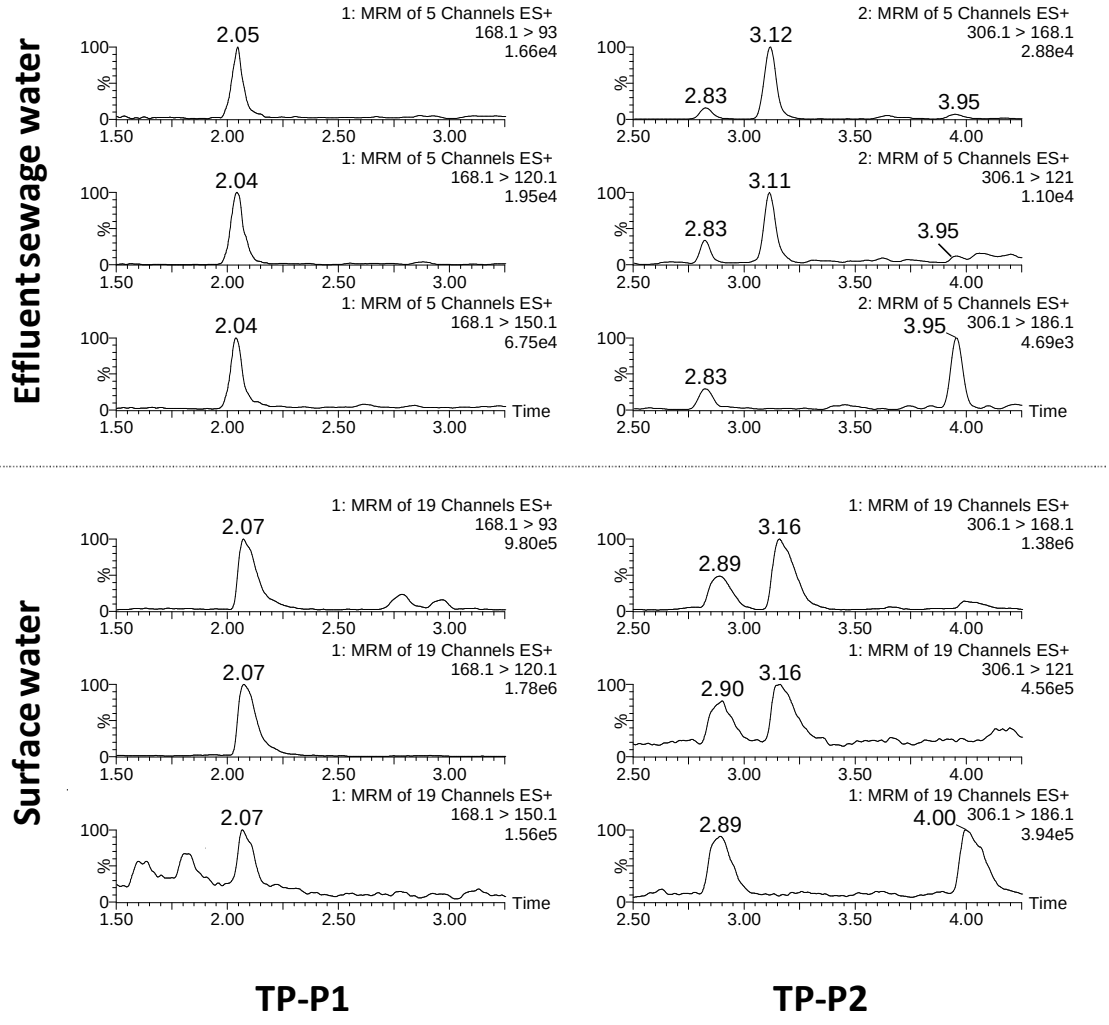


Figure 4. UHPLC-MS/MS chromatograms corresponding to the positive finding of TP-P1 and TP-P2 in (top) effluent sewage water (analyzed using TQD, December 2011) and (bottom) surface water (analyzed using TQS, April 2012). Retention times of BE were 3.75 min (TQD) and 3.84 (TQS).

In future studies, additional degradation experiments should be performed in wastewaters in order to address the presence of some of the identified TPs in influents analyzed. Moreover, reference standards of the discovered TPs are required in order to report concentration levels. Subsequently, extended monitoring studies should be set up analyzing paired wastewater and receiving surface waters. This will give more insight in the environmental fate of cocaine, its metabolites and TPs.

4. Conclusions

Data on the presence of TPs of organic contaminants in the aquatic environment are required nowadays to have a realistic overview of water quality. UHPLC–QTOF MS has been demonstrated in this work as a valuable tool for the identification of TPs of cocaine and its main metabolite BE in water. After laboratory-controlled hydrolysis, chlorination and photo-degradation experiments, the structures of several TPs have been tentatively established. The applicability of these studies has been demonstrated by analyses of sewage water (influent and effluent) and surface water where, in addition to well-known cocaine metabolites, other TPs identified in laboratory experiments have been found and reported for the first time. The relevance of TPs should not be neglected, as it might be necessary to take them into account in future monitoring studies. Other knowledge gaps, such as ecotoxicity and the effects of multiple compound exposure, need to be addressed in order to perform well-founded environmental risk assessment. This implies that lot of research is still required by environmental scientists and analytical chemists, especially when dealing with emerging contaminants.

Acknowledgements

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Supplementary data

In this section, two figures, one reporting the identification of cocaine chlorination TP-C8 (Figure S1) and the other including the identification of cocaine photo-degradation TP-P3 (Figure S2), are included to have supportive visual information on the written text.

Supplementary data to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scitotenv.2012.11.006 and in this chapter after section "References".

References

Antonelli M, Mezzanotte V, Nurizzo C. Wastewater disinfection by UV irradiation: short long term-efficiency. Environ Eng Sci 2008; 25: 363-373

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

Bijlsma L, Sancho JV, Hernández F, Niessen WMA Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MS^E accurate-mass spectra. J Mass Spectrom 2011; 46: 865 – 875

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

Binelli A, Pedriali A, Riva C, Parolini M. Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha*. Chemosphere 2012; 86: 906-911

Bisceglia KJ, Roberts AL, Schantz MM, Lippa KA. Quantification of drugs of abuse in municipal wastewater via SPE and direct injection liquid chromatography mass spectrometry. Anal Bioanal Chem 2010; 398: 2701-2712

Boleda MR, Galceran MT, Ventura F. Behavior of pharmaceuticals and drugs of abuse in a drinking water treatment plant (DWTP) using combined conventional and ultrafiltration and reverse osmosis (UF/RO) treatments. Environ Pollut 2011; 159: 1584 - 1591

Chiaia AC, Banta-Green C, Field J. Eliminating solid phase extraction with large-volume injection LC/MS/MS: analysis of illicit and legal drugs and human urine indicators in US wastewater. Environ Sci Technol 2008; 42: 8841-8848

European Monitoring Centre for Drugs and Drug Addiction. The state of the drugs problem in Europe. EMCDDA Annual Report 2010. Available from URL: http://www.emcdda.europa.eu/ publications/annual-report/2010. Accessed 10 July 2012

Farré M, Pérez S, Kantiani L, Barceló D. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. Trends Anal Chem 2008; 27: 991 - 1007

Fatta-Kassinos D, Meric S, Nikolaou A. Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research. Anal Bioanal Chem 2011; 399: 251-257

Gheorghe A, van Nuijs A, Pecceu B, Bervoets L, Jorgens PG, Blust R, Neels H, Covaci A. Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry. Anal Bioanal Chem 2008; 391: 1309-1319

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

Hernández F, Bijlsma L, Sancho JV, Díaz R, Ibáñez M. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. Anal Chim Acta 2011; 684: 96-106

Hernández F, Ibáñez M, Pozo OJ, Sancho JV. Investigating the presence of pesticide transformation products in water by liquid chromatography-mass spectrometry with different mass analyzers. J Mass Spectrom 2008; 43: 173-184

Huerta-Fontela M, Galceran MT, Ventura F. Stimulatory drugs of abuse in surface waters and their removal in a conventional drinking water treatment plant. Environ Sci Technol 2008; 42: 6809-6816

Ibañez M, Sancho JV, Pozo OJ, Hernández F. Use of quadrupole time-of-flight mass spectrometry in environmental analysis: elucidation of transformation products of traizine herbicides in water after UV exposure. Anal Chem 2004; 76: 1328-1335

Kern S, Fenner K, Singer HP, Schwarzenbach RP, Hollender J. Identification of transformation products of organic contaminants in natural waters by computer-aided prediction and high-resolution mass spectrometry. Environ Sci Technol 2009; 43: 7039-7046

Klette KL, Poch GK, Czarny R, Lau CO. Simultaneous GC-MS analysis of meta- and para-hydroxybenzoylecgonine and norbenzoylecgonine: A secondary method to corroborate cocaine ingestion using nonhydrolytic metabolites. J Anal Tox 2000; 24: 482-488

Masqué N, Marcé RM, Borull F, Cormack PAG, Sherrington DV. Synthesis and evaluation of a molecularly imprinted polymer for selective on-line solid-phase extraction of 4-nitrophenol from environmental water. Anal Chem 2000; 72: 4122–4126

Metz DH, Meyer M, Dotson A, Beerendonk E, Dionysiou DD. The effect of UV/H_2O_2 treatment on disinfection by product formation potential under simulated distribution system conditions. Water Res 2011; 45: 3969 - 3980

Morrison RT, Boyd RN. Electrophilic aromatic substitution. In: Organic chemistry, 6th ed. Prentice-Hall Inc., Englewood Cliffs (NJ), ISBN: 0-13-643669-2; 1993; p. 517-548

Pichini S, Marchei E, Pacifici R, Pellegrini M, Lozano J, García-Algar O. Application of a validated high-performance liquid chromatography-mass spectrometry assay to the analysis of m- and p-hydroxybenzoylecgonine in meconium. J Chromatogr B 2005; 820: 151-156

Postigo C, Sirtori C, Oller I, Malato S, Maldonado MI, López de Alda M, Barceló D. Solar transformation and photocatalytic treatment of cocaine in water: Kinetics,

characterization of major intermediate products and toxicity evaluation. Appl Catal B: Environm 2011; 104: 37-48

Quintana JB, Rodil R, López-Mahía P, Muniategui-Lorenzo S, Prada-Rodríguez D. Investigating the chlorination of acidic pharmaceuticals and the by-product formation aided by an experimental design methodology. Water Res 2010; 44: 243-255

Tanaka S, Lio R, Chinaka S, Takayama N, Hayakawa K. Analysis of reaction products of cocaine and hydrogen peroxide by high-performance liquid chromatography/mass spectrometry. Biomed Chromatogr 2002; 16: 390-394

Wang P-P, Bartlett MG. Collision-induced dissociation mass spectra of cocaine, and its metabolites and pyrolysis products. J Mass Spectrom 1998; 33: 961-967

Wick A, Wagner M, Ternes TA. Elucidation of the transformation pathway of the opium alkaloid codeine in biological wastewater treatment. Environ Sci Technol 2011; 45: 3374-3385

Wilson ID, Nicholson JK, Castro-Perez J, Granger JH, Johnson KA, Smith BW, Plumb RS High resolution "ultra performance" liquid chromatography coupled to oa-TOF mass spectrometry as a tool for differential metabolic pathway profiling in functional genomic studies. J Proteome Res 2005; 4: 591–598

Zuccato E, Castiglioni S, Bagnati R, Chiabrando C, Grassi P, Fanelli R Illicit drugs, a novel group of environmental contaminants. Water Res 2008; 42: 961-968

Supplementary data

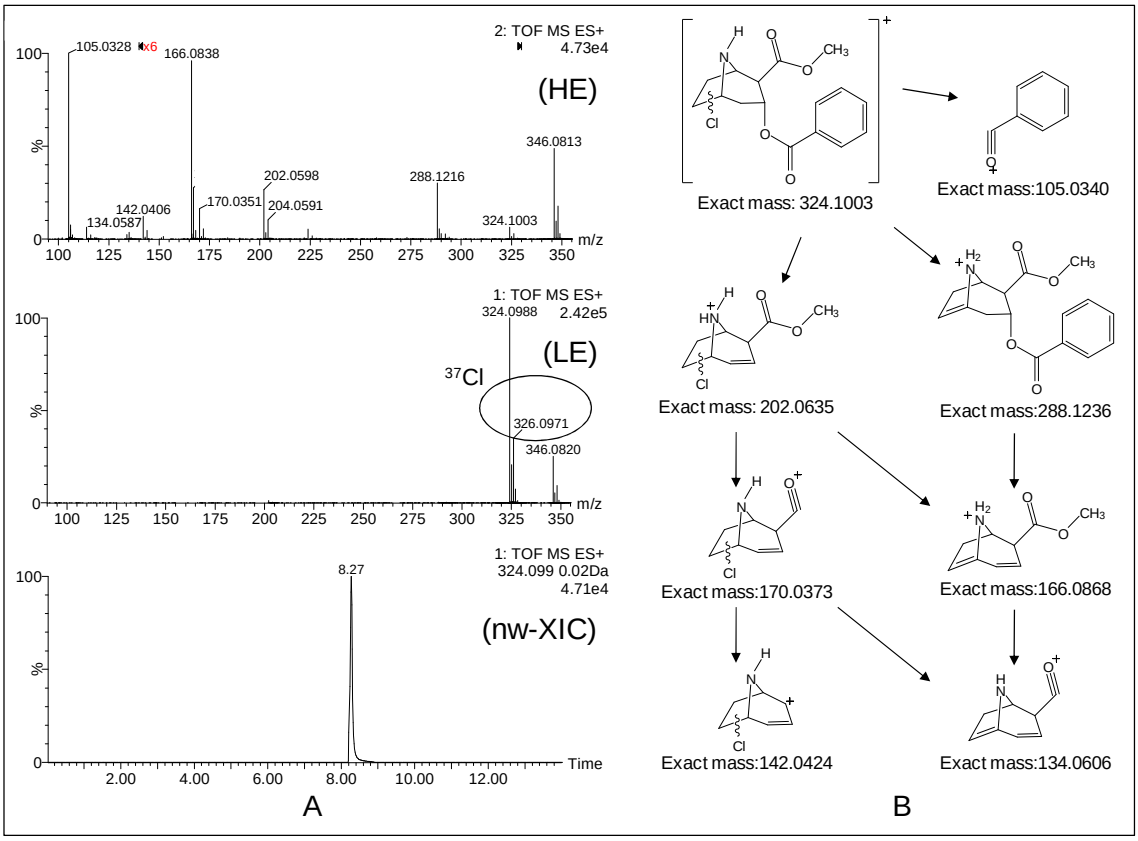


Figure S1. Detection and identification of cocaine chlorination TP-C8 by UHPLC-QTOF MS operating in MS^E acquisition mode. (A) narrow-window XIC (bottom), LE spectra (middle) and HE spectra (top) with zoom in the range *m/z* 140-340. (B) Structure proposals for $[M+H]^+$ of TP-C8 and its more abundant fragment ions.

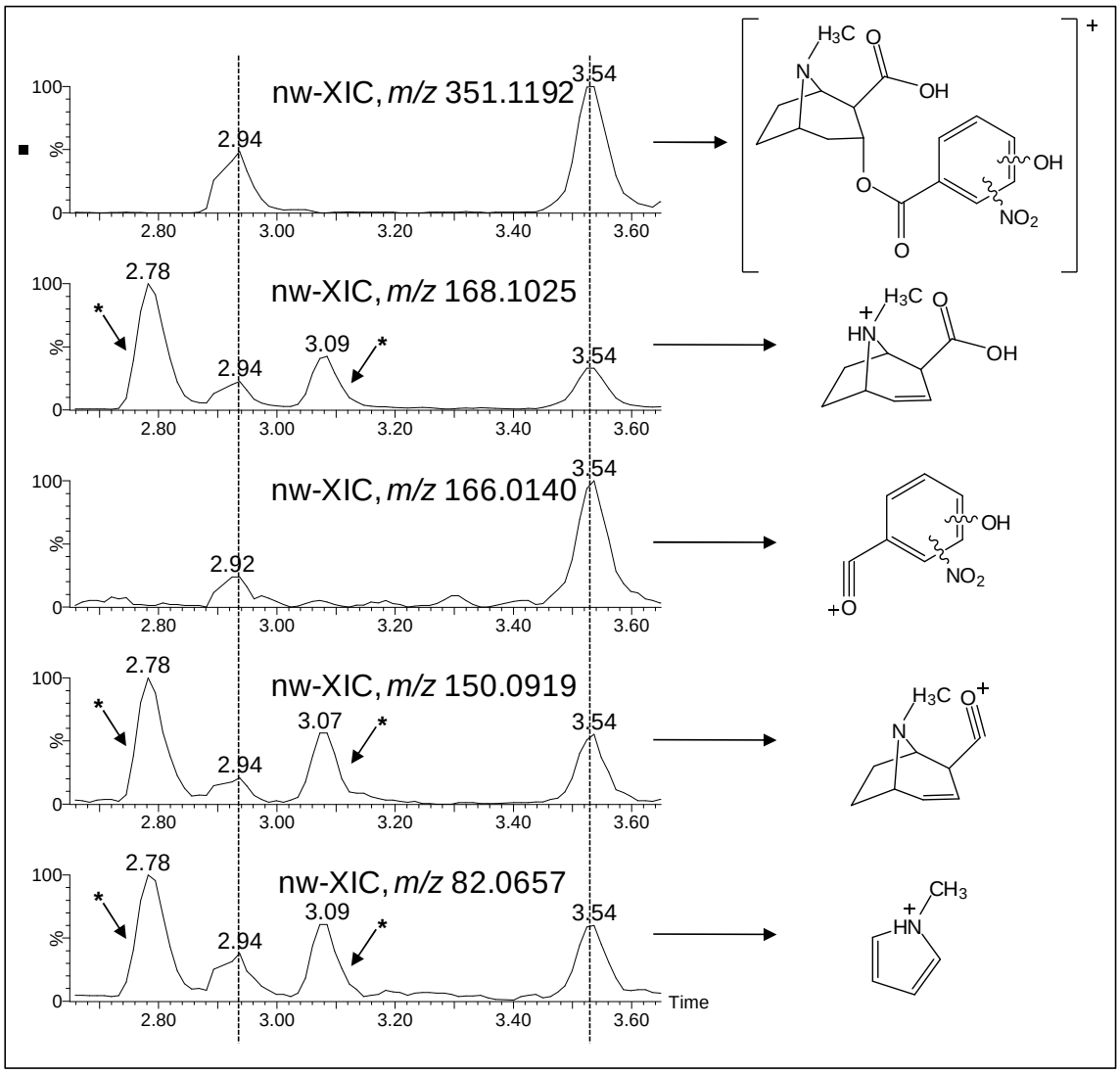


Figure S2. Detection and identification of cocaine TP-P3 by UHPLC-QTOF MS operating in MS^E acquisition mode. Narrow-window XIC of TP-P3 and various fragment ions. Structure proposals for $[M+H]^+$ of TP-P3 and its fragment ions. Notice the presence of other chromatographic peaks (marked with *), supporting that other TPs share the same fragmentation.

4.3 Discussion of the results

Fragmentation pathways of IDs and their metabolites

The use of hybrid QTOF MS makes it is feasible to record the full-scan accurate-mass product ion spectra working in MS/MS mode. Another possibility is the MS^E approach, which allows collecting simultaneously information on both (de)protonated molecules and their fragment ions, by acquiring data at low and high collision energy in a single injection. Both approaches were applied in this work to study fragmentation pathways of several classes of IDs and their metabolites (*scientific article* 6). Practical parameters, such as accurate *m/z* values, isotopic distribution patterns from the MS^E data and DBE were used to facilitate data interpretation. Furthermore, basic fragmentation concepts and rules, taking into account the structure of the parent drug, and the accurate mass data of fragment ions observed for deuterated analogues led to plausible structure proposals of fragment ions. In most cases, accurate mass information allowed the confirmation of the identity of fragment ions previously proposed by other authors, commonly based on nominal mass measurements. In some particular cases, accurate-mass data was essential to understand the fragmentation and/or to discard structures suggested in the literature. All information in combination with the high quality of the analytical data provided by QTOF MS helped to elucidate structures and to complete fragmentation pathways with high reliability and confidence.

An illustrative example of ***the power of accurate mass*** was demonstrated when elucidating a fragment ion with *m/z* 105 of the amphetamine-type stimulants MDA, MDMA and MDEA. This ion had been interpreted in the literature as being a structure with an elemental composition corresponding to $C_7H_5O^+$ (calculated *m/z* 105.0340). However, our accurate-mass data (*m/z* 105.0703) suggested an elemental composition of $C_8H_9^+$ (calculated *m/z* 105.0704, mass error 0.1 mDa) and consequently corresponding to a different structure of this fragment ion (**Fig. 4.1**). In addition, data of the labeled analogues proofed this interpretation to be correct.

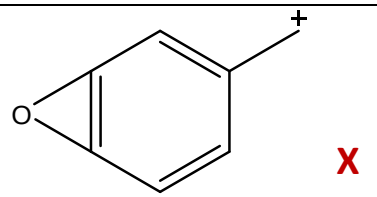
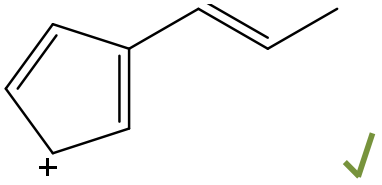
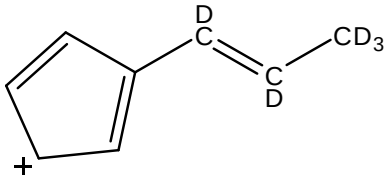
Structure fragment with *m*/z 105	Structure labeled fragment with *m*/z 110
 X *m*/z 105.0340	X five D-labels not possible in this structure
 ✓ *m*/z 105.0704	 ✓ *m*/z 110.1018

Figure 4.1. Structure of fragment ion with *m*/z 105 of the amphetamine-type stimulants MDA, MDMA and MDEA.

Thus, accurate mass data of the ***deuterated analogues*** served as a confirmation tool. Yet, comparison of mass spectra of the labeled and unlabeled compound was essential for the identification of fragment ions, especially the less abundant ones, and for understanding the fragmentation route. In particular, D-labeling demonstrated to be important in elucidation of the fragmentation pathways of THC-COOH and several opiates.

Regarding opiates, fragment ions at the lower end of the spectra have generally been ignored in the literature as their formation is complex and involves multiple neutral losses and/or losses of larger neutral fragments. At this point, it was helpful to perform additional experiments using a QqQ instrument. By performing ***precursor-ion scan*** (see Figure 1.7, of the general introduction) of the more abundant low-*m*/z fragment ions, more insight was obtained in the fragmentation pathways of opiates.

As stated above, full-scan accurate-mass "*product ion*" spectra can be obtained by QTOF MS working in MS^E mode. An advantage of this mode is that during MS^E acquisition, the quadrupole (Q) operates in wide-band transition mode in such a way

that all ions are transmitted into the collision cell, providing useful information regarding ***isotopic distribution pattern***. For example, a characteristic isotopic pattern corresponds to ^{35}Cl and ^{37}Cl, where ^{37}Cl is present at around 30% of that of ^{35}Cl. This information together with the accurate mass was very helpful to confirm the fragmentation pathway of ketamine ($C_{13}H_{16}NOCl$) [Wang *et al.* 2005], as this ID contains one chlorine atom, and various fragment ions could be linked to the presence or loss of chlorine.

The information obtained from the study on fragmentation pathways of several classes of IDs and their metabolites (*scientific article 6*) was very useful for the selection of specific transitions (*scientific articles 1 and 2*). Furthermore, several characteristic fragments could be included in the customized accurate-mass database developed in *scientific articles 3 and 4* (see also figure 3.2 of chapter 3). The insertion of these fragment ions allowed confirmation of identified compounds during screening, giving more confidence to the obtained results.

Spectra obtained in MS/MS and MS^E mode were in general rather similar, demonstrating similar capabilities for both approaches. Then, the use of MS^E was considered more suitable and easy to apply for a wide-scope screening, and for identification and elucidation of TPs, as no precursor ion needs to be selected in the first quadrupole.

Investigation of TPs

After consumption of IDs and subsequent excretion, the parent compounds and/or their metabolites enter WWTPs. Several treatment processes can be applied in order to remove these compounds. In most WWTPs, primary treatment (sedimentation) and secondary treatment (activated sludge) are performed followed by conventional tertiary processes such as filtration, nitrogen and phosphorus removal or grit removal. Occasionally, additional disinfection processes, such as ozonation, chlorination or UV irradiation are applied by some WWTPs. During these treatment processes IDs and their metabolites may be completely or partly removed and/or transformed into disinfection by-products (*i.e.* TPs) that consequently may be released in receiving surface waters. Furthermore, IDs and metabolites which end up in surface waters, due to incomplete

removal, may be exposed to natural sunlight and produce photo-degradation products. The occurrence of TPs in the environment is increasingly recognized as an important issue, as some show higher persistence and/or toxicity than the parent drug. However, most authors have focused research on the parent compounds and/or known metabolites, regardless of the resulting TPs. The main limitations associated with the analysis of TPs are that many have not yet been identified, that they are most likely formed at low concentrations and that there is a lack of analytical standards.

Cocaine is second-highest consumed ID in Europe [EMCDDA 2012]. Concentrations of cocaine and BE found in influent sewage water of Spain are generally in the same order of magnitude than most pharmaceuticals [Gracia-Lor *et al.* 2012; Ibáñez *et al.* 2013b], and BE uses to be the most abundant compound among all IDs/metabolites investigated in sewage water. Hydrolysis, chlorination and UV irradiation, occasionally applied by local WWTPs, can lead to the formation of TPs. Therefore, a study of degradation products of cocaine and BE in the aquatic environment was conducted in *scientific article 7* in order to have more information on the fate and behaviour of these two major compounds in the aquatic environment.

All degradation experiments were performed using blank surface (river) water, without any adjustment of pH and temperature, in order to simulate realistic environmental conditions. Spiked (either with cocaine or BE) and blank surface water were kept in darkness at room temperature to study hydrolysis. After 15 days cocaine was mainly transformed into BE and some degradation was observed for BE (**Fig. 4.2**). However, it is noteworthy that biodegradation might also occur, as matrix components present in the surface water may affect degradation.

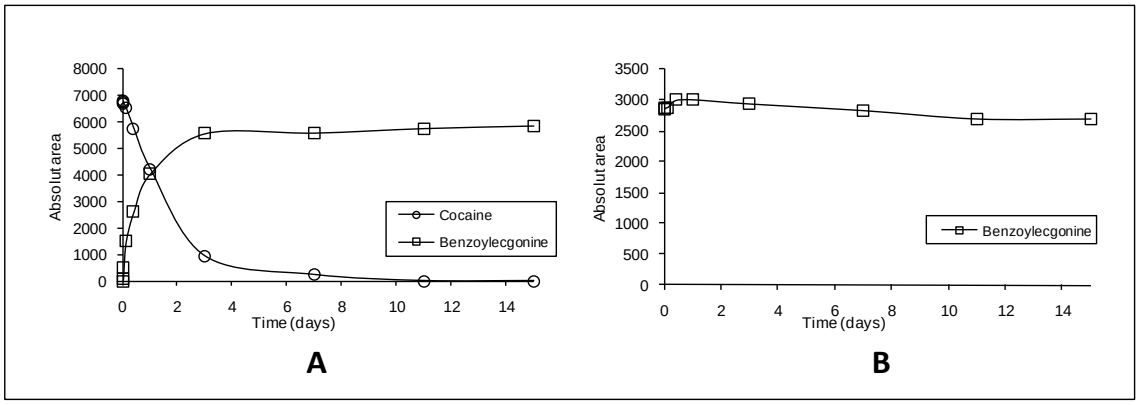


Figure 4.2. Hydrolysis of A: cocaine and B: benzoylecgonine

High chlorine concentrations (8 mg/L) and a mercury lamp (254 nm) were used, to simulate the chlorination and UV irradiation conditions employed by the WWTPs. Similarly, a solar simulation system equipped with a xenon arc lamp and a special solar light filter was used to provide a wavelength distribution that is close to natural sunlight and constant irradiance, avoiding the variances caused by geographical situation, seasonal or meteorological conditions. Biodegradation experiments were not performed because no adequate material was available at our laboratory.

Experiments under these laboratory-controlled conditions in combination with QTOF MS^E were employed to identify and elucidate TPs. The LE function was used to screen for precursor ions, and accurate mass of fragments observed in the HE function were used to discard potential chemical formulas and to obtain the most plausible structures of TPs. Knowledge of the fragmentation pathway of cocaine and its metabolites previously investigated was also very helpful [Wang *et al.* 1998; Bijlsma et al. 2011 - *scientific article* 6]. The most abundant and common fragment of cocaine and most of its metabolites can be assigned to the neutral loss of benzoic acid (122 Da) and the remaining fragment ions mainly involve subsequent loss of methanol or water.

Identification and elucidation of TPs of cocaine and BE

The Waters MetaboLynx XS software (an application manager within MassLynx) was used to process data obtained from degradation studies. This software compares two

LC-MS data files, one corresponding to the spiked analyte sample and the other corresponding to the blank/control sample, and detects, identifies and reports differential ions/chromatographic peaks that would correspond, in principle, to TPs (**Fig. 4.3**). MetaboLynx XS proved to be highly useful for the investigation of both expected and unexpected TPs.

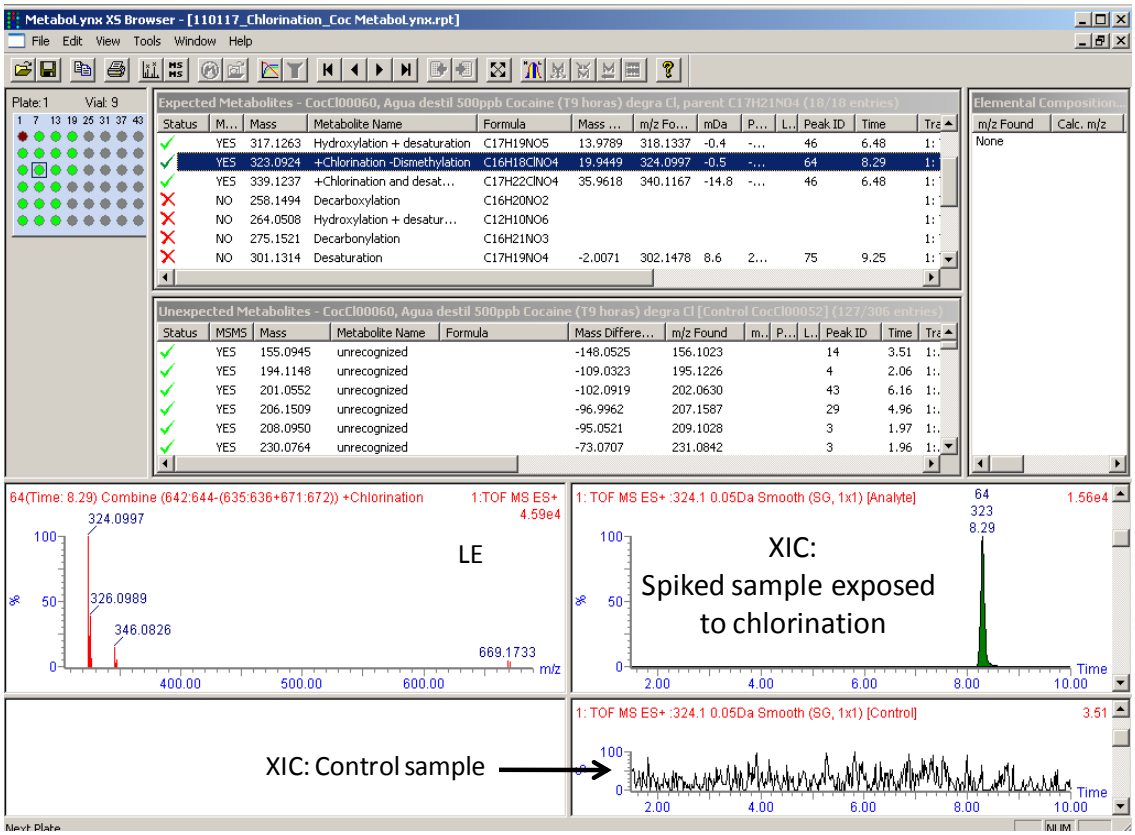


Figure 4.3. Snapshot of Metabolynx XS browser; detection of cocaine TP-C8, elucidation can be found in the supporting information of ***scientific article 7*** (***Figure S1***).

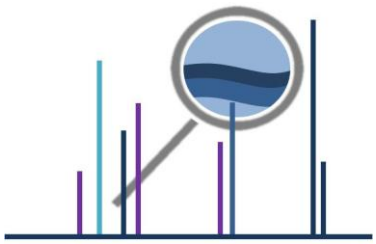
Acquisitions were performed in centroid, under positive and negative ion mode. For all compounds detected by MetaboLynx, the accurate mass of (de)protonated molecules was determined on the basis of averaged spectra obtained in the survey scan. Then, possible elemental compositions of the peaks of interest were calculated using elemental composition calculator, within the MassLynx software, with a maximum deviation of 2 mDa from the measured accurate mass. Considering the elemental

composition and structure of cocaine (C17H21NO4), the maximum and minimum parameters were restricted as follows: C 0–25, H 0–40, N 0–4, and O 0–10. In chlorination experiments, the number of chlorine atoms was selected on the basis of the observed isotopic pattern. The applied DBE filter was set between 0.5 and 10. In those cases where more than one elemental composition was obtained, neutral losses were investigated in both HE and LE functions, trying to reduce the number of plausible elemental compositions. Furthermore, TPs of cocaine and/or BE shared their core structures and often had the same fragment ions, which was useful for elucidation of TP structures. Similar workflow has been used in other studies [Ibañez *et al.* 2006; Boix *et al.* 2013]

With all the information and possibilities offered by hybrid QTOF MS (and the software), the tentative identification of the detected TPs was feasible, even without reference standards. Up to sixteen TPs of cocaine were detected; three were well known metabolites, and they could be confirmed by their reference standards. Regarding the cocaine metabolite BE, which has been found at high concentrations in wastewaters, up to ten TPs were found, including one known metabolite. In total 9 TPs, not previously reported as metabolites, could be tentatively identified. Data of the TPs detected and identified can be found in ***tables 2 and 3*** and ***figure 1 of scientific article 7***.

After elucidation and tentative identification of the TPs formed during laboratory-controled degradation experiments, several water samples were screened for the identified TPs using UHPLC-MS/MS (QqQ) and an off-line SPE procedure, for sample pre-concentration and clean-up. Besides known metabolites, four photo-degradation TPs (of which three were isomers) were detected in several influent and effluent wastewaters and surface waters. ***Figure 4 of scientific article 7*** shows positive findings of these four TPs in effluent and surface water. The fact that the three SRM transitions acquired and that the retention times were in agreement with the TPs identified in degradation experiments give high confidence to these findings, despite the fact that reference standards for unequivocal confirmation were not available.

Although further research is required, this work illustrates the interest to include these TPs in multi-residue methods and monitoring programs to have a wider overview on the presence of cocaine and its metabolites in the aquatic environment and on the problematic associated to this highly consumed ID. Moreover, now that new TPs have been identified, further studies of toxicity and persistence should be performed to complete our knowledge of the environmental fate of cocaine and BE.



CHAPTER 5

Occurrence of illicit drugs in the aquatic environment

5.1 Introduction

The amounts of illicit drugs consumed worldwide are comparable with those of pharmaceuticals, as millions of individuals are current users of amphetamines, cocaine, opioids, cannabis and other drugs. Like pharmaceuticals, illicit drugs are excreted, after their consumption, as unchanged parent compound and/or as metabolites in consumers urine and feces, and enter the urban sewage networks. Subsequently, these compounds are partially removed during wastewater treatment and consequently amounts of these compounds are released into receiving waters.

The presence of IDs in the aquatic environment have been receiving increased interest, since Jones-Lepp *et al*. from the EPA reported for the first time in the peer-reviewed literature the detection of methamphetamine and MDMA, in treated sewage effluents in the United States in 2004 [Jones-Lepp *et al.* 2004], and Zuccato and co-workers from the Mario Negri Institute in Italy reported the occurrence of cocaine in the Po river in 2005 [Zuccato *et al.* 2005]. IDs and their metabolites are currently often measured in WWTP effluents and surface waters from Australia, Europe and North America [Huerta-Fontela *et al*. 2008a; Kasprzyk-Hordern *et al*. 2008; Bartelt-Hunt *et al*. 2009; van Nuijs *et al*. 2009; Postigo *et al*. 2010; Irvine *et al*. 2011]. Recently, IDs have been recognized as new group of emerging contaminants in the environment [Zuccato *et al*. 2008a; Richardson and Ternes 2011].

The presence of emerging contaminants in environmental waters has become an issue of major concern in the scientific community. Several reports on the occurrence IDs and their degradation or transformation products have been reported. Concentrations were generally found lower in effluents than influents, suggesting degradation or sorption of most of these substances in WWTPs [Huerta-Fontela *et al*. 2008b; Boleda *et al*. 2009; Terzic *et al.* 2010]. However, significant amounts of some IDs and metabolites were still present in effluents and can therefore consequently end up in the receiving water bodies (*i.e*. surface water) and even in finished drinking water produced from surface water [Huerta-Fontela *et al*. 2008a]. The reported concentrations of IDs in surface water are in general low [Huerta-Fontela *et al*. 2008a; Kasprzyk-Hordern *et al*. 2008; Bartelt-Hunt *et al*. 2009; van Nuijs *et al*. 2009; Postigo *et al*. 2010; Vazquez-Roig et al. 2010]. Nevertheless, data on environmental levels are still limited and much more research is needed for a better understanding of the environmental occurrence, transport, fate, and exposure of IDs and their (often) active metabolites. Large monitoring exercises are therefore important to be carried out more frequently. Moreover, information on possible toxicological implication on biota and potential synergistic effects through combined exposure to multiple compounds are still unknown and is also required in order to perform a well-founded environmental hazard and risk assessment in the near future.

The quantitative measurement of IDs in urban influent wastewater (*i.e*. diluted and pooled community samples from the inlet of a WWTP) can also provide useful and objective information of patterns and trends of ID use at a community level [Zuccato *et al*. 2005]. The levels of drug residues in the sewage reflect the amount of a particular drug that has been consumed by the population served by the sewer network under investigation. In some cases, consumption is studied through the presence of a specific drug biomarker, such as in the case of THC-COOH, the main urinary metabolite of cannabis. The approach is often referred to as "sewage-based epidemiology" (SBE) and, besides the measured drug concentrations, uses the water flow rate and population size to back-calculate and estimate drug consumption. Generally, the consumption data derived from wastewater analysis is in agreement with existing prevalence data. SBE is considered as complementary to existing drug estimation

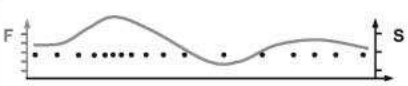
techniques as it provides evidence based and near real-time estimates of ID consumption. It can be used to estimate local, national and international consumption, to monitor drug use changes in time, identify changing trends or new habits, and identify the use of new drugs [Zuccato *et al*. 2008b; Banta-Green *et al.* 2009; van Nuijs *et al*. 2011b; Thomas *et al*. 2012; Reid *et al*. 2014]. This new approach is being applied in many countries around the world, as in Australia, Belgium, Canada, Croatia, Czech Republic, Finland, France, Italy, the Netherlands, Norway, Spain, Sweden, Switzerland, United Kingdom, and the USA [Metcalfe *et al*. 2010; Castiglioni *et al*. 2011; Daughton 2011; Irvine *et al*. 2011; van Nuijs *et al*. 2011a]; and it has put in practice in this chapter yielding promising results [Thomas *et al*. 2012 – *scientific article a*; Ort *et al*. 2014 – *scientific article c*]. As demonstrated in these studies, SBE is an approach with great potential, but still in its early stages of development and it requires further optimization and standardization.

The SBE approach requires the application of advanced analytical methodologies, and it has not been feasible until recently when technological advances in analytical chemistry have facilitated the development of sensitive and accurate methodologies, based on chromatography and mass spectrometry, able to detect these substances at trace levels (ng/L) in complex matrices, such as urban wastewater. However, SBE has a strong multidisciplinary character and requires close collaboration between analytical chemists, wastewater engineers, biologists, pharmacologists, epidemiologists and policy makers. In the last few years, several shortcomings from the different scientific disciplines have been identified [Ort *et al*. 2010; Baker and Kasprzyk-Hordern 2011; van Nuijs *et al*. 2011a; Baker *et al*. 2012] and uncertainties have been tested and addressed [Castiglioni *et al*. 2013 – *scientific article b*] in order to reduce over- or under-reporting of drug concentrations and produce more reliable and comparable estimates of ID use.

In continue, some of the main uncertainty issues related to sample collection, compound stability, and analyses are briefly discussed. The results are important for formulating recommendations on these aspects.

Sample collection: Ort *et al*. (2010) critically reviewed the sampling procedure in wastewater systems. This is of particular interest, because it can have large impact on measured concentrations of IDs and metabolites in wastewater. Different sampling strategies can be applied as illustrated in **Table 5.1**. All composite sampling modes use the same methodology: a mixture of individual sub-samples, collected at regular intervals (based on time or flow), made by an automatic sampling device normally during 24-h. The sampling frequency, referred to as the interval between two sub samples (aliquots), can differ from one methodology to another, but is extremely important to obtain a representative sample. Close collaboration with local WWTP staff are imperative to gather detailed information on catchments properties, sewage system characteristics, WWTP inlet data, sampling mode and frequency, and flow rate meter properties.

Table 5.1: Different sampling modes (from Ort *et al*. 2010)

Sampling mode		Short description (see *Sampling Guide* to find out which sampling mode is suitable in which situation).	Illustration (F=Flow in sewer, S=Sampling volume)
Continuous	flow-proportional	Divert a side stream, proportional to the flow in the sewer	
	constant	Divert a constant side stream from the sewer	
Discrete	time-proportional	Take a constant sample volume at constant time intervals	
	flow-proportional	Make sample volume proportional to the flow in the sewer taking them at constant time intervals	
	volume-proportional	Take a constant sample volume at variable time intervals, after a certain volume of wastewater has passed the sampling point	
	grab sample	Take one (or a number of) grab sample	

Compound stability: The stability of the selected IDs in the samples is an important issue. Degradation or even formation of drug residues can occur during (a) transport in the sewers (*i.e.* from the place of excretion to the place of sampling at the WWTP) and (b) collection and storage of the water samples. Stability experiments have been carried out under different pH (2 to 8) and temperature (-20 to +20 °C) conditions [Castiglioni *et al*. 2006; Gheorge *et al*. 2008; Gonzalez-Mariño *et al*. 2010]. However, only few authors performed experiments for a wide range of IDs and their metabolites at temperature (20 °C) and pH (7.5) conditions relevant for sewer systems [Baker and Kasprzyk-Hordern 2011; van Nuijs *et al*. 2012b]. The results addressed some stability issues, and recommendations on ideal conditions for the storage of water samples were formulated, yet further and more detailed research was also recommended. A general conclusion was that water samples can be stored for weeks when frozen at -20 °C.

Analysis: The focus of this PhD thesis clearly lies on analytical chemistry. The reliable determination of low concentrations of IDs and their metabolites in complex water samples is an analytical challenge. Accurate data on analyte concentrations in wastewater are the basis of the subsequent calculation of ID loads and consumption, and can be used to estimate removal efficiencies of WWTPs by comparison of IDs concentrations in influent and effluent wastewater. Accurate quantification and replicate analysis are obviously required, but confirmation of analyte identity is of outstanding relevance to avoid reporting false positives and/or false negatives. To this aim, the application of quality criteria based on acquisition of accurate mass data in HRMS, or several transitions in LR MS/MS methods, and on their specificity, retention times and ion intensity ratios is required to get a safe identification. Methodologies for quantitative and qualitative analysis need to be fully-validated using analytical recognized and accepted quality criteria (e.g. SANCO/12495/2011; 2002/657/EC). Furthermore, the participation in inter-laboratory studies is highly useful as it provides relevant information on the performance and may disclose systematic errors. Accordingly, the application of quality controls, both external and internal, is a key issue to guarantee the quality of data reported.

It is worth noting that most limitations identified and recognized in the SBE approach, such as those related to sampling, analyses and stability, also affect the quality of data in wastewater effluents and surface waters. A reliable determination of IDs in these matrices permits a better and more realistic knowledge on their removal by WWTPs and on the possible impact on environmental waters.

5.2 Scientific articles

The determination of IDs and their metabolites in the water cycle contributes to the understanding of the potential impact of these compounds on the aquatic ecosystem, but the results can also provide information on drug use and trends. In the first two studies presented this chapter, the occurrence of IDs in wastwater samples from Spain and the Netherlands is being discussed. Based on the data obtained, information on short-time changes in drug use at local level could be derived. The performances of WWTPs were also evaluated, and subsequent discharges to receiving waters were estimated. In a third study, a screening of IDs was performed in samples of the Dutch watercylce, including wastewater, surface water and drinking water.

The first work (*scientific article 8*) presents a study on the occurrence and behavior of 11 IDs and metabolites in sewage water systems, employing the validated analytical protocol discussed in Chapter 2 of this thesis (Bijlsma *et al*. 2009 - *scientific article 1*). The selected IDs included several amphetamines, cocaine and its metabolites, and the main metabolite of cannabis. A comprehensive data set was obtained by analyzing the IDs daily in influent and effluent wastewaters from three WWTPs, over three different weeks. The WWTPs selected were sited along the Spanish Mediterranean coast (Castellón province, Valencia region) and represent towns of different size, with appreciable variations in the population in the summer period. To complete this data set, monitoring was conducted during an international pop/rock festival, an interesting aspect within this study. The data obtained gave information on short-time changes in drug use, especially during the celebration of the music festival and, by comparing concentration data of both influent and effluent wastewater an evaluation on the removal efficiency of treatment processes applied by each WWTP could be made. Subsequently, weekly loads discharged into the Mediterranean Sea could be calculated from effluent data.

In a second work (*scientific article* 9), the presence of 24 IDs and pharmaceuticals with potential for abuse in sewage waters was studied for the first time in the Netherlands. Influent and effluent wastewaters from WWTPs serving four cities of different size and the international airport of Schiphol were analyzed using LC coupled to LTQ FT Orbitrap MS,

employing the validated analytical protocol discussed in Chapter 3 of this thesis (Bijlsma *et al*. 2013 - *scientific article 5*). Samples were taken during a whole week. Daily variances of drug loads and removal efficiencies were calculated for each drug and WWTP individually. This manuscript got recognised by the Society of Environmental Toxicology and Chemistry – Europe (SETAC) and received the Eurofins Best Publication award 2012.

In a third work (*scientific article 10*), a screening of 34 drugs of abuse and their relevant metabolites in the Dutch watercylce was performed. Urban wastewater (influent and effluent), surface water of the rivers Rhine and Meuse, and drinking water that is produced from those rivers, were analysed by four different laboratories using fully in-house validated methods. In this way data, reported for several compounds could additionally be confirmed by other laboratories, giving extra confidence to the results obtained. Toxicological relevance for environment and human health were evaluated through concentrations found in surface water and finished drinking water, respectively.

It is important to emphasise that *scientific articles* 9 and 10 are the result of a close collaboration between different institutions. The contribution of PhD candidate to these manuscripts is explained in Chapter 1, section 1.4.

The results of the three studies presented in this chapter have been published in:

- *Science of the Total Environment* 487, **2014**, 703 – 709
- *Chemosphere* 89, **2012**, 1399 – 1406
- *Water Research* 47, **2013**, 1848 – 1857

Chapter 5.2.1, scientific article 8

Occurrence and behavior of illicit drugs and metabolites in sewage water from the Spanish Mediterranean coast (Valencia region)

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Occurrence and behavior of illicit drugs and metabolites in sewage water from the Spanish Mediterranean coast (Valencia region)

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Graphical Abstract

Highlights

- The presence of illicit drugs in sewage waters of three municipals is investigated
- Daily variances of three times one-whole week were studied
- One sampling week coincided with one of the largest European music festivals
- Removal efficiencies were calculated for each drug and treatment plant individually
- Weekly discharges of illicit drugs to the aquatic environment were estimated

Abstract

In this work, a study on the occurrence and behavior of illicit drugs and metabolites in sewage water systems has been made. A comprehensive dataset was obtained by analyzing illicit drugs daily in influent and effluent waters from three sewage treatment plants (STPs), over three different weeks. To complete this dataset, monitoring was conducted during an international pop/rock festival, an interesting facet within this study. The STPs selected were sited along the Spanish Mediterranean coast (Castellón province, Valencia region) and represent towns of different sizes, with appreciable variations in the population in the summer period. Illicit drug concentrations in the influents were low, except during the celebration of the music festival, when the levels of cocaine, benzoylecgonine, amphetamine, MDA and MDMA increased. Comparing the influent and effluent concentration data allowed the rough estimation of the removal of illicit drugs and metabolites by each STP. Removal efficiencies were estimated between 75 and 100% for most of the analytes under investigation. The loads discharged into the aquatic ecosystem were also calculated from effluent data. Weekly discharges of drugs and metabolites via effluent sewage waters presented values commonly below 10 g for each individual drug, with the exception of benzoylecgonine, which usually exceeded this level. The increase in population and drug consumption during the music event led to a notable increase in the weekly discharges, reaching values up to 406 g of MDMA and 122 g of benzoylecgonine.

Keywords

Illicit drugs, sewage water, STP removal, environmental loads

1. Introduction

Illicit drugs enter the sewage system, unaltered or as metabolites, most commonly after their consumption and excretion, but also after illegal discharges as consequence of police interventions (Thomas et al., 2012). Studies on the occurrence of these compounds in influent waters from sewage treatment plants (STPs) provide useful information on drug use and consumption trends at local, national and international levels (Nefau et al., 2013; Thomas et al., 2012; van Nuijs et al., 2011a and Zuccato et al., 2008a). The possibility of performing daily sample analysis makes it feasible to have real-time and objective information on drug use of a community. Some recent studies report spatial and temporal variations in the occurrence of illicit drugs in wastewater, including holidays and "control" periods (Lai et al., 2013a), or performing analysis along one year (Harman et al., 2011 and van Nuijs et al., 2011a). Standard sampling devices are normally used to obtain 24-h composite samples, but polar organic chemical integrative samplers (POCIS) have also been used (Harman et al., 2011).

Some limitations of sewage water analysis such as the estimation of population sizes contributing to the samples in each catchment area, drug excretion and analytes stability in the sewage system have been discussed in the recent literature (Castiglioni et al., 2013; Thomas et al., 2012 and van Nuijs et al., 2011b). Despite the limitations and uncertainties associated with sewage water analysis, interesting trends in drug use have been observed, with prominent increase related to particular celebrations (Harman et al., 2011; Lai et al., 2013a; Lai et al., 2013b and van Nuijs et al., 2011a). However, the removal of these compounds by STPs during these high peaks of drug usage was not evaluated.

The efficiency of the treatment processes in the STPs can be estimated from drug concentrations in influent and the corresponding effluent, taking into account the residence time of water in the STP (Bijlsma et al., 2012 and Postigo et al., 2010). According to the literature, illicit drugs and their metabolites may be released into aquatic environments due to their insufficient elimination in STPs. Thus, these compounds have been detected in natural surface waters (Baker and Kasprzyk-Hordern, 2011; Berset et al., 2010; Van der Aa et al., 2013 and Zuccato et al., 2008b),

including Spanish rivers (Huerta-Fontela et al., 2008; Pedrouzo et al., 2011 and Postigo et al., 2010). Concentrations found are generally low (sub ppb level) and therefore no short-term direct environmental or human health effects are expected. However, very little is known about aquatic ecotoxicology and the effects of illicit drugs on biota; therefore, long-term (chronic) effects and possible effect of combined exposure are not ruled out (van der Aa et al., 2013). Large monitoring campaigns reporting concentration data for illicit drugs in environmental waters might give more and better insight on the environmental fate of these compounds. Therefore, it is important to evaluate the removal efficiency for illicit drugs in the STPs and the possible discharge of these compounds to the aquatic environment not only under normal conditions but also in particular situations, when increased concentrations of illicit drugs are expected, i.e. festivities, music events, festivals, etc.

In this work we report data on occurrence of 11 illicit drugs and metabolites in influent and effluent waters from three STPs, representing towns of different sizes, along the Spanish Mediterranean coast (province of Castellón; Valencia region). Three sampling periods of one week each were spread over one year (June, July, January and/or April), including one week which coincided with a big music festival. In total, 126 wastewater samples were taken, making the dataset comprehensive and allowing us to set up the following objectives: (i) to have information on the occurrence of illicit drugs in sewage waters in the catchment area, including some periods when tourism increases appreciably, (ii) to study the removal of each compound by three STPs serving different cities, (iii) to estimate the weekly load discharge towards the aquatic ecosystem through concentration data in the effluents, and (iv) to evaluate the influence of an important music event on the amount of illicit drugs in wastewaters and on the removal efficiency of the STP. The information obtained in this study reveals short-time changes in drug use at local level and provides useful information on the suitability of the treatment processes used by the STPs.

2. Materials and methods

2.1. Sample collection

The three STPs studied were located along the Spanish Mediterranean coast and served the communities of Benicasim, Castellón and Burriana (see Graphical abstract). The number of people connected to each STP was taken from census information procured in 2009, and corresponded to 170.600 (Castellón), 40.283 (Burriana) and 15.564 (Benicasim). It is worth noting that the population of Benicasim drastically increases in summer, particularly during the festival included in this investigation. Influent and effluent sewage water samples were collected from the three STPs daily during one week in summer (June 2008) and one week in winter (January 2009). In addition, samples were also collected from Benicasim STP during one week in July 2008 (coinciding with this important music event) and from Castellón and Burriana during one week in April 2009. Samples consisted on 24-h composite samples which were taken using a time-proportional sampling mode (1 L, every hour). They were collected at refrigerated conditions (4 °C) in high-density polyethylene (HDPE) bottles and 1 L homogenized 24-h sample was transported to the laboratory directly after taking the last aliquot. Upon reception in the laboratory (within 15 min), samples were immediately stored in the dark at − 20 °C until analyses (within 1 week) to minimize degradation of analytes.

2.2. Target analytes

Illicit drugs were selected as a function of their use in our area, focusing the research on amphetamines, cocaine and cannabis. Their most relevant metabolites were also selected for this study. The target analytes were the following: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA, or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, cocaethylene, benzoylecgonine, norbenzoylecgonine, norcocaine, and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH), a relevant metabolite of Δ^9-tetrahydrocannabinol (cannabis). Isotope-labeled compounds were used as surrogate internal standards (ILIS) for quantification: amphetamine-d_6, methamphetamine-d_5 MDA-d_5, MDMA-d_5, MDEA-d_5, cocaine-d_3,

cocaethylene-d_8, benzoylecgonine-d_3 and THC-COOH-d_3. More details on chemicals and materials can be found elsewhere (Bijlsma et al., 2009).

2.3. Sewage water treatment

All STPs investigated are equipped with conventional activated sludge secondary treatment. The main differences between them refer to their water treatment capacity and additional treatment processes. STP Benicasim applied an extra tertiary nitrogen and phosphate removal, whereas STP Burriana applied a disinfection step by chlorination and STP Castellón had an additional physical treatment (incl. grit removal). The average daily flow of each STP was Castellón 47412 m^3; Burriana 12056 m^3; and Benicasim 5253 m^3. For the three STPs, the average residence time is between 12 and 24 h.

2.4. Analytical methodology

Sample treatment and specific information on instrument operating conditions, both chromatographic and spectrometric, and on method validation can be found elsewhere (Bijlsma et al., 2009). Briefly, 50 mL of effluent or 50 mL of five-time diluted influent sewage water were spiked with a mixed ILIS solution and the pH was adjusted to 2.0 with formic acid. Solid phase extraction (SPE) was performed using Oasis MCX cartridges. After elution with 2% ammonia solution in methanol, the extracts were evaporated and reconstructed in 1 mL of 10% methanol aqueous solution. The final sample extract (20 µL) was injected directly into the UHPLC–MS/MS system. Chromatographic separation of the compounds was achieved using an Acquity UPLC BEH C_{18} column and an optimized gradient using methanol: water (5 mM ammonium acetate, 0.1% formic acid). A TQD triple quadrupole mass spectrometer was operated in positive ionization mode, where three SRM transitions for each target compound were acquired. All data were acquired and processed using MassLynx v 4.1 software (Waters, Manchester, UK).

2.5. Calculation of removal efficiencies

Removal efficiencies were estimated by comparing effluent concentrations (Ce) from day (x + 1) with influent concentrations (Ci) from day (x), thus considering an average

residence time of 24 h. Efficiencies (E) were calculated as $E = (1 - (Ce(x + 1) / Ci(x)) \times 100\%$ (Bijlsma et al., 2012). For each STP and compound, average removal efficiencies were estimated from the daily values.

3. Results and discussion

3.1. Sample analysis and quality assurance

The determination of illicit drugs in influent and effluent sewage waters requires advanced analytical methodologies to be able to provide accurate concentration data in these complex matrices. At present, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is the workhorse in this field (Baker and Kasprzyk-Hordern, 2011; Bijlsma et al., 2009; Pedrouzo et al., 2011 and Thomas et al., 2012), due to its excellent performance in terms of sensitivity, selectivity and robustness.

In this work, we applied the methodology described by Bijlsma et al. (2009). Data reported are the mean concentration of 2 measurements (replicates) based on 2 individual extractions. In every sequence of analysis, samples were injected in between two calibration curves prepared in solvent. Two quality control samples (QCs), consisting of water samples with the lowest concentrations expected (i.e. samples collected from Tuesday to Thursday), spiked at the limit of quantification (LOQ) and 10 × LOQ levels, were also analyzed in every sequence of sample analysis. The sequence was considered satisfactory when recoveries ranged from 70 to 120% for each analyte. Quantification criteria and confirmation parameters, such as the ion ratio between recorded transitions, retention time and the accepted deviation, were applied before reporting positive findings (Commission Decision 2002/657/EC, 2002 and SANCO/12495/2011 (supersedes document no. SANCO/10684/2009), 2011).

The analyte-ILIS of each illicit drug, except for norcocaine and norbenzoylecgonine, was used to compensate for matrix effects and for possible errors related to sample treatment. In general, matrix effects resulted in ionization suppression and, as expected, were stronger for influent wastewater. Amphetamines and especially THC-COOH were the analytes more affected by matrix effects.

Although some concentration data obtained in this work were below the lowest level validated (Bijlsma et al., 2009), they could be reported as the signal-to-noise ratio was ≥ 10 (i.e. the statistical limit of quantification). Furthermore, the reliable quantification of THC-COOH in influent samples was problematic, and only few data could be reported due to the strong matrix effects observed (ionization suppression) for this analyte. On the contrary, in effluent samples, which were much cleaner and less affected by matrix effects, THC-COOH could be quantified in several occasions, even if it was present at concentrations lower than in influent.

3.2. Occurrence of illicit drugs in influent waters

The occurrence of illicit drugs and metabolites in influent waters from the three STPs (i.e. communities) under study is shown in **Fig. 1** and **Fig. 2**. In general, cocaine and its main metabolite benzoylecognine (BE) were the most abundant compounds in the three weeks of monitoring, suggesting a notable consumption of cocaine in the area under study. This is consistent with the annual report of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), which reported levels of cocaine use in Spain above the European average using statistical data of 2008 (EMCDDA, 2010). Cannabis is another drug with high prevalence in Spain (EMCDDA, 2010). This might be explained by the fact that cocaine and cannabis enter Europe largely through the Iberian Peninsula (EMCDDA, 2010). Thus, the availability of these drugs is probably higher, allowing easier access and thereby increasing consumption of these drugs rather than others. The average estimated consumption of these illicit drugs in Spain (data reported for Barcelona, Castellón, Valencia and Santiago de Compostela) through sewage water analysis is approximately 1000 mg cocaine/day/1000 inhabitants (based on BE loads) and 100 mg THC-COOH/day/1000 inhabitants (Thomas et al., 2012).

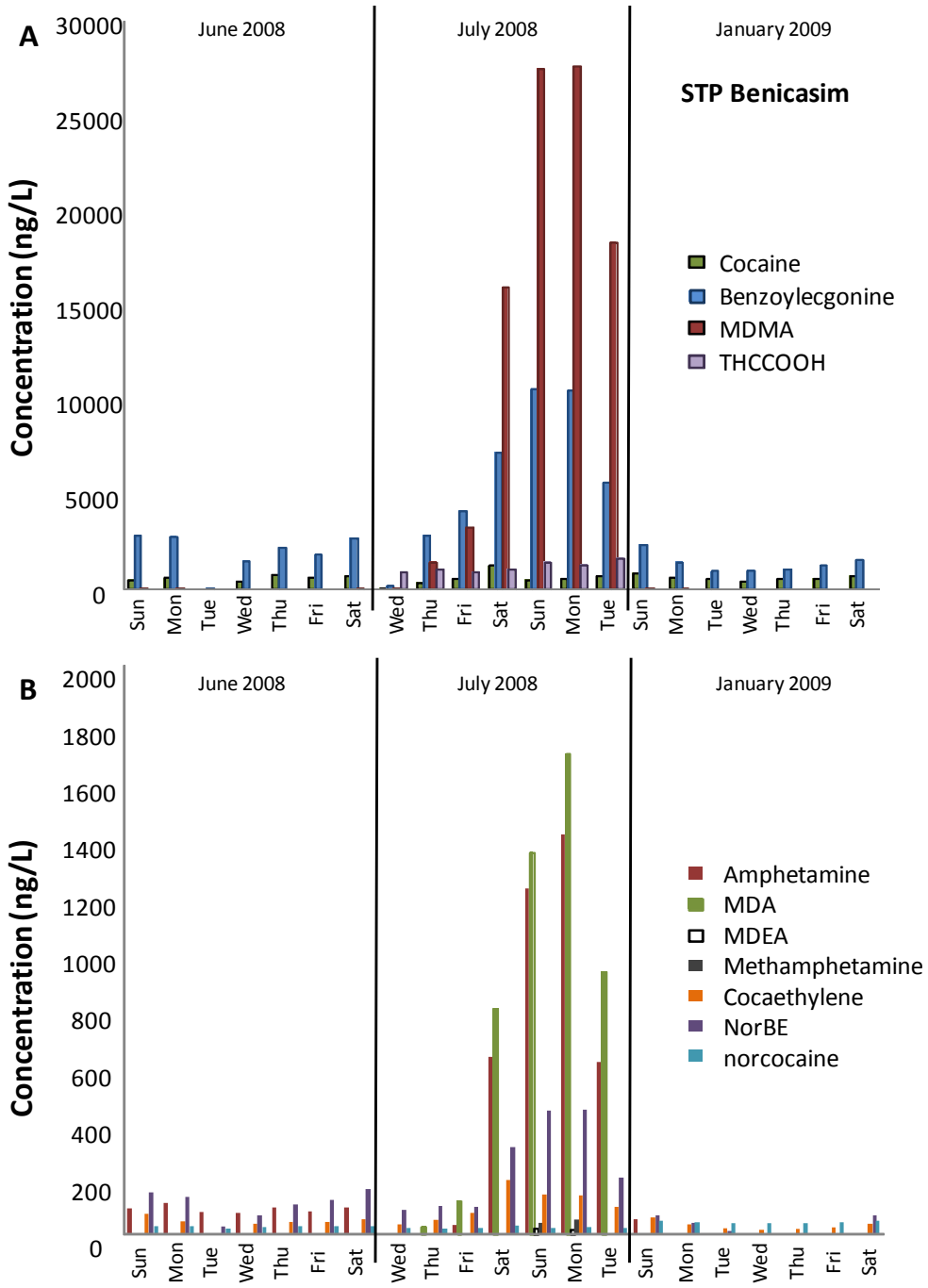

Figure 1. Weekly profile of illicit drug and metabolite concentrations (ng/L) in influent sewage waters from the Benicasim STP in June, July (music event) and January. Top: Major compounds. Bottom: Minor compounds.

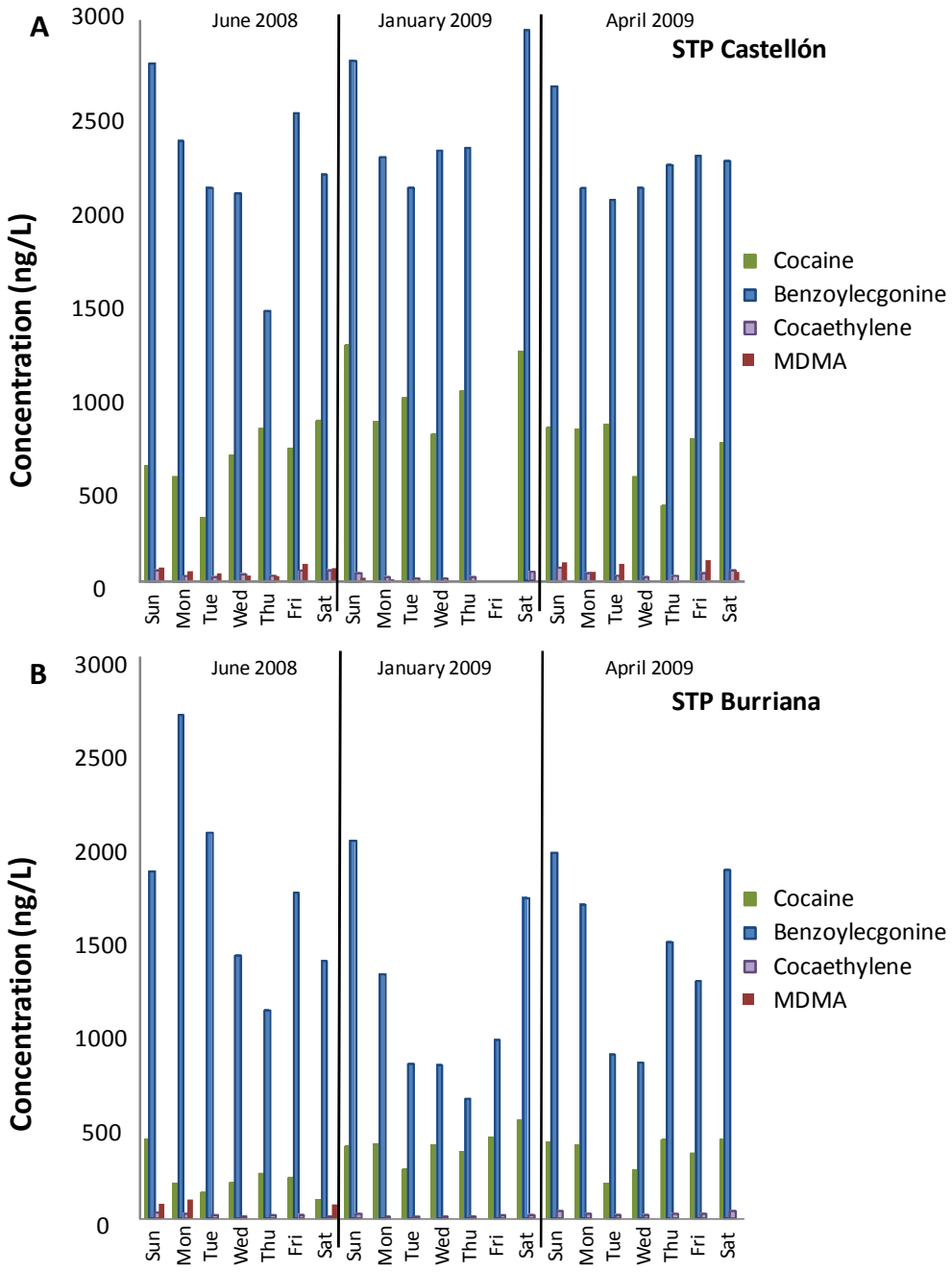

Figure 2. Weekly profile of concentrations (ng/L) of major illicit drugs and metabolites in influent sewage waters from the STP of Castellón and Burriana in June, January and April.

Fig. 1 shows the occurrence of illicit drugs in sewage water from Benicasim. The STP commonly serves only this town, which has a population of 15,564 inhabitants. However, it is a known holiday destination, mainly for Spanish families and pensioners, and therefore its population increases drastically during summer. Furthermore, it hosts one of the largest Spanish and European annual pop, rock and electronic festivals, visited by approximately 40,000 young music fans. In 2008, the festival was held from July 17 to 20 and the STP also treated the wastewater obtained from the portable toilets used at the festival terrain. It is important to point out that the disposal of waste from these portable toilets occurred before the composite sampler.

In this work, sewage samples from Benicasim were collected during one whole week of June 2008, July 2008, and January 2009. Slightly higher concentrations of BE and amphetamine were found in June (summer) compared with January (winter), which would reflect the increase in the consumption of these drugs in the holiday period. These findings are in agreement with the works from Harman et al. (2011) and Lai et al. (2013a), who also reported increased use of amphetamine, methamphetamine, cocaine and MDMA in the holiday period in Oslo region (Norway), and a vacation area in Queensland (Australia), respectively.

The significant increase in concentration of MDMA, THC-COOH, cocaine and BE observed during the week of July 2008, which coincided with the music festival is worth noticing (**Fig. 1, top**). Other compounds such as amphetamine, MDA and the cocaine metabolites, norbenzoylecgonine and cocaethylene were also found at higher concentrations during this period, although their levels were by far below BE, the main metabolite of cocaine (**Fig. 1, bottom**). THC-COOH could only be quantified in the influent samples from July (concentration range 918–1638 ng/L). Several factors may explain the non-detection of this compound in most influent samples. Obviously, the analytical limitations, mainly the poor sensitivity, play a key role which makes the quantification of THC-COOH troublesome, and also sorption to solids may be greater (Harman et al., 2011), due to its different physico-chemical properties (lower polarity) compared with other illicit drugs.

The most interesting finding was the extraordinary increase in the party drug MDMA (ecstasy) during the music festival. This drug was barely detected during the weekends of June and January (**Fig. 1, top**). The fact that MDA was also found in the influent samples of July is interesting. MDA is a minor metabolite (7% of a dose) of MDMA (Castiglioni et al., 2008), and MDMA was found at high concentrations in the influents (above 27 µg/L in some of the samples; see **Fig. 1**). This might explain the finding of MDA in influents. However, MDA is also available on the illicit market. Therefore, the presence of this compound might be due to consumption of MDA itself, or it might proceed from the consumption or transformation in the sewage system from MDMA. The analytical method applied to the analysis of the water samples did not allow distinguishing between the enantiomeric forms of MDA. Enantiomeric profiling of chiral drugs has proven to be very helpful. Thus, an enrichment of MDA with S(+)-enantiomer in urine and subsequently in wastewater would, for example, indicate that MDA is present due to MDMA abuse and not direct MDA use (Kasprzyk-Hordern and Baker, 2012).

As outlined above, data obtained on illicit drugs in influents during the festival is interesting. The prominent increases in occurrence of illicit drugs were in agreement with data reported regarding similar events. For example, high levels of MDMA and cocaine use were observed on New Year's Eve (Lai et al., 2013a and van Nuijs et al., 2011a). Similarly, higher MDMA concentrations could be linked to graduation festivities (Harman et al., 2011), a big dance party (van der Aa et al., 2013), and an annual Australian music festival (Lai et al., 2013b) celebrated within the catchment areas. And although methamphetamine was slightly lower, Gerrity et al., 2011 reported data suggesting an elevated use of cocaine during the Super Bowl weekend compared to an average weekend.

Weekly profiles for illicit drug concentrations in STP sited at the other two towns, Castellón and Burriana, are shown in **Fig. 2** for three periods of the year (summer, winter and spring). The populations of Castellón and Burriana are relatively constant, although there are some fluctuations as many families move to the beach area (e.g. Benicasim) in summer. This means that the number of inhabitants in July and August is lower than the rest of the year. As previously mentioned, BE was the most abundant compound in

all samples studied, maintaining a rather constant relative abundance with cocaine during all studied period. The average cocaine/BE ratio in all sewage waters included in this work was 0.30 ± 0.14, which is higher than the ratio ≤ 0.1 reported for human metabolism, but in agreement with ratios (0.1–0.7) obtained from wastewater analysis of 21 STPs (Castiglioni et al., 2013). Moreover, the highest concentrations of these compounds were commonly found on weekends, as illustrated by the data from samples collected from Saturday to Monday. The increased cocaine consumption during the weekend compared to weekdays was also observed by others (Thomas et al., 2012 and van Nuijs et al., 2011a). Generally, concentrations of illicit drugs in influent waters from Castellón were higher than that from Burriana. The population of Castellón is 5-times higher than that of Burriana and this fact seems to lead to higher concentrations of illicit drugs and metabolites in the influent waters. This is in accordance with other studies, where higher consumption was related to larger cities (van Nuijs et al., 2011b and Banta-Green et al., 2009), surely not only because of the higher population but mainly because of the lifestyle associated to big cities.

In addition to these results, our recent data from Castellón sewage waters (March 2011 and April 2012) show concentrations in influents about the same magnitude. Average weekly concentrations in 2011 and 2012 for cocaine were 400 ng/L and 450 ng/L, and for BE 1000 ng/L and 1400 ng/L, respectively. Furthermore, recent improvements in the analytical methodology for THC-COOH, especially better method sensitivity and lower limits of detection and quantification, have facilitated the determination of THC-COOH in influent waters from Castellón, with average weekly concentrations of 300 ng/L and 600 ng/L in 2011 and 2012, respectively. For this purpose, the use of new-generation more sensitive LC–MS/MS instrumentation was essential (Bijlsma et al., 2013).

3.3. Occurrence of illicit drugs in effluent waters: Removal efficiency of STPs

The efficiency of the treatment processes of STPs can be estimated from illicit drug concentrations in influents and from their corresponding effluents, taking into account the residence time of water in the plant (Bijlsma et al., 2012 and Postigo et al., 2010). In this study, we applied the same approach for estimating the removal efficiency of each STP, which requires the analysis of illicit drugs in influent and effluent samples. It is

important to notice that removal was calculated considering an average residence time of 24 h. As the residence time of the STPs varied between 12 and 24 h, there will be some uncertainties associated with the calculations presented in this work. Therefore, data given in this paper must be taken as a rough, albeit useful, estimation of the removal efficiency of the STPs due to the large number of samples collected (data from 21 influent and 21 effluent samples for each STP).

Fig. 3 shows the average removal efficiencies for several illicit drugs in three STPs. Nearly all values were higher than 60% with a few exceptions that mostly correspond to the STP of Benicasim during the festival event of July. The removal of amphetamine, cocaine and its main metabolite BE was ≥ 75% for all STPs, which compares well with the range of 81–99% reported by Baker and Kasprzyk-Hordern (2013); Huerta-Fontela et al. (2008); Kasprzyk-Hordern et al. (2008) and Repice et al. (2013). Removal of the other cocaine metabolites, cocaethylene, norbenzoylecgonine (norBE) and norcocaine was ≥ 75% for all STPs, except for norBE by the Castellón STP. Baker and Kasprzyk-Hordern (2013) reported removals with the use of activated sludge of 42% and 68% for norbenzoylecgonine and cocaethylene, respectively. MDMA presented high variability with values from 34% to 89%. This is in agreement with data reported by Bijlsma et al. (2012), who also found highly variable removal efficiencies for MDMA in five Dutch STPs. Since MDA and THC-COOH were not commonly detected in influent samples, the removal for these compounds could only be estimated for the STP serving Benicasim during the festival week.

In general, the removal efficiencies were not much different over the three weeks studied along the year (June, January and April). This implies that the applied treatment does not seem to be highly affected by meteorological conditions (i.e. temperature, which ranges on average from 5 °C in January to 30 °C in June at this latitude) and that bioactivity/degradation was similar in summer and winter.

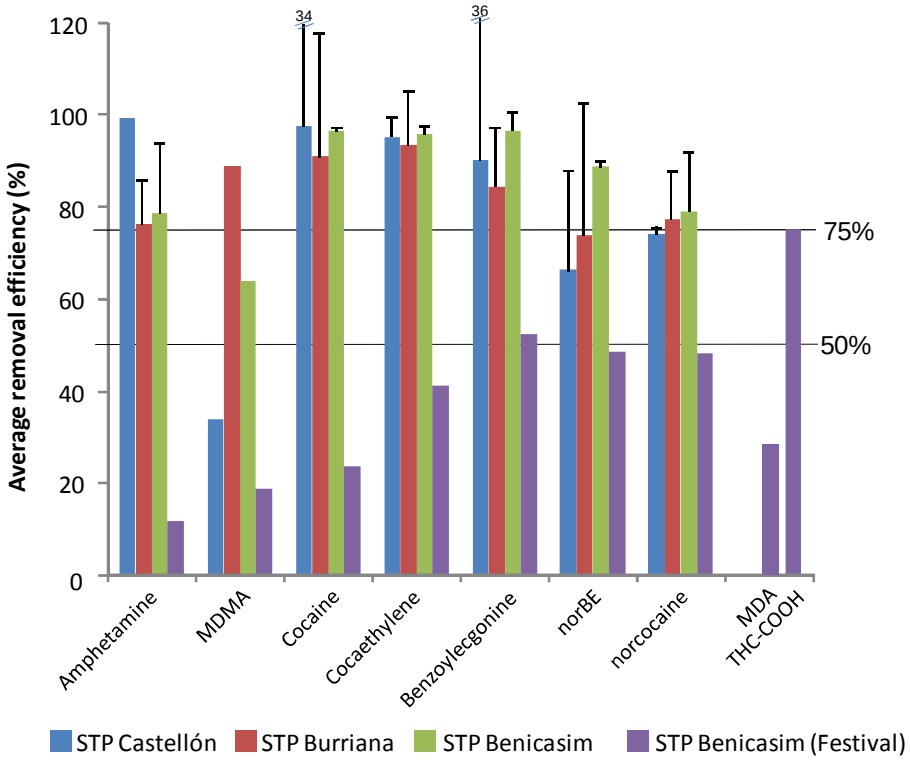

Figure 3. Average removal efficiencies (%) for illicit drugs in different STPs.

The STP of Benicasim, designed to treat sewage water of a small community (approx. 15,000 inh.) showed satisfactory removal (75–100%) for all compounds, except MDMA. However, some difficulties were observed during the music festival (**Fig. 3**). As stated in the previous section, concentrations of illicit drugs in influent waters during this festival were considerably higher (see **Fig. 1**), as a consequence of the drastic increase in population and consumption of illicit drugs linked to these events. It seems that the drastic increase in population during the festival week, added to the common increase of tourists in summer, creates some difficulties in the treatment processes of sewage water.

More recent data obtained from Castellón STP (2011 and 2012) have enabled a new estimation of removal efficiencies for cocaine, BE as well as THC-COOH. The removal of cocaine and BE was consistent with data from previous years, with efficiencies higher than 80%. The removal efficiency for THC-COOH could not be estimated in our analysis

from 2008 and 2009 samples due to the above-mentioned difficulties in determining this cannabis biomarker in influent samples. However, with an improved analytical methodology, we were able to use our data from 2011 and from 2012 to estimate the THC-COOH removal, which was found to be higher than 90% in both years.

As previously stated, the removal efficiency was estimated using concentrations of analytes in solely sewage waters. The lower levels commonly found in effluents are assumed to be a result of removal in the STP, due to microbial degradation, or other transformation processes. However, the analysis of suspended particular matter (SPM) has also been suggested to prevent under-reporting. The analysis of both sewage waters (influent and effluent) and SPM provides a better estimation and more realistic knowledge on removal and environmental impact of compounds by STP systems, as removal from wastewater does not necessarily mean degradation (Baker et al., 2012 and Baker and Kasprzyk-Hordern, 2013). For most illicit drugs included in this study, less than 5% was estimated to be present in SPM (Baker et al., 2012), thus potential under-reporting was assumed to be low. THC-COOH was not included in that study, but partition to SPM might be greater for this compound compared with the other selected drugs, owing to its less polar character. Furthermore, the stability of illicit drugs in sewage is an important issue. Amphetamine, methamphetamine, MDA, MDEA, MDMA and THC-COOH are generally stable up to 72 h at 4 °C (Castiglioni et al., 2006). However cocaine, norcocaine and cocaethylene are not stable under these conditions (decrease > 10%), and can be transformed into BE and norBE through chemical hydrolysis (Castiglioni et al., 2013). In this study, the bias due to cocaine transformation is expected to be relatively low as the estimated mean residence time of wastewater in the sewers investigated is 3 h and the conditions for sampling and sample storage minimize degradation of the selected compounds. The highest uncertainty within this study seems to be related to the sampling (Ort et al., 2010). In this work, sampling error was estimated to be relatively high (around 30%) (Castiglioni et al., 2013) due to the time proportional sampling interval used (every hour). This uncertainty has been reduced for the studies performed in 2011 and 2012 by taking a sample every 15 min.

As demonstrated in this work, the results obtained from the analysis of sewage water give useful information on trends of drugs usage from the population and the removal of these compounds by STPs. However, for the correct interpretation of these results it is important to take into account some of the uncertainties associated with the determination of illicit drugs in sewage (Castiglioni et al., 2013 and van Nuijs et al., 2011b).

3.4. Environmental loads

Weekly loads (g) of illicit drugs and metabolites discharged via sewage effluents into the aquatic ecosystem were estimated from daily concentrations of each compound detected in effluent water (ng/L) and the daily flow of effluent water discharged along the week (**Table 1**). Loads (g/week) of illicit drugs (MDMA, cocaine, BE, cocaethylene and THC-COOH) from effluent waters of Castellón could be updated for 2011 and 2012, and are also included in **Table 1**.

The effluents of the three STPs studied are discharged into the Mediterranean Sea. Occasionally, effluents of the Castellon STP are also used for irrigation. The discharges for each individual compound were generally below 10 g per week, with only a few exceptions, mainly BE, with discharges up to 49 g/week in Castellón (2012). The main discharges predominantly corresponded to cocaine and its metabolites, which were released by all three STPs. This might be expected as cocaine (and BE) was the most abundant drug found in sewage waters. Although the determination of THC-COOH in influent waters was troublesome, it could be determined on several occasions in effluents, which are less prone to matrix interferences when using LC–MS/MS. Consequently, the environmental loads of THC-COOH could be estimated. Weekly discharges of THC-COOH were 6.4 g and 3.2 g for Castellón (June 2008 and April 2012, respectively) and 9.9 g for Benicasim (July 2008). Although concentrations of MDMA in influent waters were not very high (sub-ppb level), this compound was frequently detected in the effluents. Similarly to THC-COOH, this fact might be due to less matrix interferences affecting LC–MS/MS analysis in this type of samples making detection of illicit drugs easier, or owing to the low removal of MDMA by the STPs (see **Fig. 3**). Weekly discharges of MDMA were mostly below 10 g in the three STPs evaluated.

Table 1. Weekly loads (g) and range of daily loads (g) (in brackets) of illicit drugs discharged in the environment via effluent waters by the three STPs studied.

STP	Amph.	MDA	MDEA	MDMA	Metamph.	Cocaine	Cocaethylene	BE	NorBE	Nor Coc	THC-COOH
CS0806 (a)	0.10 (0-0.10)	-	-	9.86 (1.08-2.17)	0.09 (0-0.09)	5.65 (0.4-1.58)	1.15 (0-0.46)	5.50 (0-1.72)	9.07 (0.98-1.67)	1.25 (0.10-0.31)	6.44 (0-6.44)
CS0901	- (b)	-	0.09 (0-0.09)	2.17 (0-0.74)	-	6.87 (0-2.22)	-	8.23 (0-4.12)	14.78 (1.85-3.14)	2.54 (0-0.56)	-
CS0904	-	-	-	-	-	1.64 (0.16-3.21)	-	10.87 (0.67-2.78)	8.04 (0.71-1.55)	1.94 (0.24-0.30)	-
CS1103	-	na (c)	na	11.73 (0-2.99)	-	6.02 (0.24-1.55)	2.43 (0-0.49)	12.5 (0-5.15)	na	na	-
CS1204	-	na	na	4.56 (0.35-1.35)	-	4.08 (0.14-1.36)	na	49.32 (5.12-8.18)	na	na	3.20 (0-0.81)
BU0806	0.21 (0.01-0.10)	-	-	0.88 (0-0.32)	-	0.54 (0.04-0.14)	-	9.68 (0.66-2.38)	2.46 (0.14-0.64)	0.22 (0-0.06)	-
BU0901	-	-	-	0.30 (0-0.14)	-	4.11 (0.39-0.94)	0.27 (0-0.07)	27.55 (1.73-6.52)	3.31 (0.20-0.84)	0.71 (0.08-0.12)	-
BU0904	-	-	-	-	-	1.65 (0.18-0.31)	-	12.02 (1.29-2.34)	3.35 (0.33-0.74)	0.64 (0.06-0.12)	-
BE0806	0.63 (0.07-0.11)	-	0.12 (0-0.06)	0.68 (0.05-0.22)	0.32 (0.04-0.06)	0.44 (0.02-0.20)	-	0.56 (0.054-0.132)	0.27 (0.02-0.06)	0.26 (0.04-0.04)	-
BE0807 (d)	12.97 (0.02-7.18)	28.34 (0.15-12.30)	0.07 (0-0.07)	405.96 (1.05-145.79)	0.08 (0-0.04)	16.21 (0.05-7.55)	2.61 (0.02-0.15)	121.67 (0.15-61.16)	3.62 (0.13-1.01)	0.41 (0.03-0.10)	9.91 (0-3.76)
BE0901	-	-	-	0.70 (0.04-0.21)	-	0.67 (0.08-0.11)	0.06 (0-0.02)	2.33 (0.18-0.47)	0.30 (0-0.05)	0.18 (0.02-0.03)	-

a. CS Castellón. BU Burriana. BE Benicasim. CS0806 means: Castellón, June 2008
b. –: no loads could be estimated as the illicit drugs were not detected in any of the influent samples analyzed
c. na: not analysed
d. Music event

A notable increase in weekly discharges was observed for Benicasim in July, as a consequence of the much higher levels of illicit drugs in sewage waters. This was expected as there was a strong increase in population and drug consumption during this week. The highest weekly discharges towards the aquatic ecosystem corresponded to MDMA and BE, which is in agreement with the high consumption of ecstasy and cocaine during the festival. Loads for cocaine, MDA and amphetamine were also higher during this week, but in all cases below 30 g.

Overall, the amounts of illicit drugs discharged into the environment reported in this work are of the same order than those reported by van der Aa et al. (2013), who estimated daily discharges ranging from 1 to 5 g of BE and 1 to 10 g of MDMA. The only exception in their study (80 g/day of MDMA) coincided with a big dance party on the day before sampling, similarly to what we observed in the present work during the festival week of Benicassim.

The lack of aquatic ecotoxicological data for narcotic substances makes it difficult to do proper environmental risk assessment. Environmental risk characterization ratios (RCRs) were calculated by van der Aa et al. (2013), in order to evaluate the potential risk associated with the presence of illicit drugs in the aquatic ecosystem. Measured environmental concentrations (MEC) found in surface water were divided by the predicted no effect concentrations (PNECs) and for values < 1 no potential risk to the aquatic environment was assumed. PNECs derived by the Ecological Structure Activity Relationships (ECOSAR) modeling were given for methamphetamine (2.30 µg/L), MDMA (2.70 µg/L), cocaine and BE (both 4.90 µg/L) (van der Aa et al., 2013). Although the authors of that manuscript recognized the limitations and uncertainties of this approach, it may give an indication of possible environmental effects. In the present work, we did not perform analysis of surface/sea water, but from the measured concentrations in effluents and considering the PNEC values, no short-term environmental risk might be expected under the treatment and normal circumstances applied in the STPs under study. In the future, the analysis of surface waters and biota might give useful information on the potential impact of illicit drugs in the aquatic environment.

4. Conclusions

The results obtained in this study from analysis of sewage waters gave valuable information of illicit drugs use in three locations on the Spanish Mediterranean coast. Moreover, as both influent and effluent samples were analyzed in three one-week periods, it was feasible to evaluate the efficiency of treatment process applied by the STPs. In general, the removal of illicit drugs by the three STPs was satisfactory (mostly above 75%) under normal circumstances, which limited the discharge of these emerging contaminants towards receiving environmental waters. In one case, during a large music festival, where the population and drug consumption dramatically increased, there was a notable increase in illicit drug concentrations in influents. This was also reflected in higher analyte levels in the effluents and in the loads to the aquatic environment. One interesting aspect is the usefulness of the methodology applied on the detection of short-time changes in drug use at local level and the effects on STP processes, and consequently on the potential impact on the aquatic environment.

Acknowledgements

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References

Baker DR, Kasprzyk-Hordern B. Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionization tandem mass spectrometry. J Chromatogr A 2011; 1218: 1620 - 31.

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

Baker DR, Očenášková V, Kvicalova M, Kasprzyk-Hordern B. Drugs of abuse in wastewater and suspended particulate matter – further developments in sewage epidemiology. Environ Int 2012; 48: 28 - 38.

Banta-Green C, Field J, Chiaia AC, Sudakin DL, Power L, de Montigny L. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater. Addiction 2009; 104: 1874 - 80.

Berset J-D, Brenneisen R, Mathieu C. Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography – Electrospray tandem mass spectrometry (HPLC-MS/MS). Chemoshpere 2010; 81: 859 - 66.

Bijlsma L, Sancho JV, Pitarch E, Ibáñez M, Hernández F. Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater. J Chromatogr A 2009; 1216: 3078 - 89.

Bijlsma L, Emke E, Hernández F, de Voogt P. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. Chemosphere 2012; 89: 1399 - 406.

Bijlsma L, Beltrán E, Boix C, Sancho JV, Hernández F. Improvements in analytical methodology based on ultra-high-performance liquid chromatography-tandem mass spectrometry for the sensitive determination of illicit drugs and their metabolites in urban wasterwater. Anal Bioanal Chem (2013) (submitted for publication).

Castiglioni S, Zuccato E, Crisci E, Chiabrando C, Fanelli R, Bagnati R. Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography - tandem mass spectrometry. Anal Chem 2006; 78: 8421 – 9.

Castiglioni S, Zuccato E, Chiabrando C, Fanelli R, Bagnati R. Mass spectrometric analysis of illicit drugs in wastewater and surface water. Mass Spectrom Rev 2008; 27: 378-394.

Castiglioni S, Bijlsma L, Covaci A, Emke E, Hernández F, Reid M, Ort C, Thomas KV, van Nuijs ALN, de Voogt P, Zuccato E. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers. Environ Sci Technol 2013; 47: 1452 - 60.

Commission Decision 2002/657/EC of 12 August implementing Council Directive 96/23/EC concerning performance of analytical methods and the interpretation of results. Off J Eur Commun 2002; L221/8.

European Monitoring Centre for Drugs and Drug Addiction. The state of the drug problem in Europe, Annual report. Luxembourg: Office for Official Publications of the European Communities; 2010.

Gerrity D, Trenholm RA, Snyder SA. Temporal variability of pharmaceuticals and illicit drugs in wastewater and the effects of a major sporting event. Water Res 2011; 45: 5399 – 411.

Harman C, Reid M, Thomas KV. In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage. Environ Sci Technol 2011; 45: 5676 - 82.

Huerta-Fontela M, Galceran MT, Ventura F. Stimulatory drugs of abuse in surface waters and their removal in a conventional drinking water treatment plant. Environ Sci Technol 2008; 42: 6809 - 16.

Kasprzyk-Hordern B, Baker DR. Enantiomeric profiling of chiral drugs in wastewater and receiving waters. Environ Sci Technol 2012; 46: 1681 – 91.

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res 2008; 42: 3498 – 518.

Lai FY, Bruno R, Hall W, Gartner C, Ort C, Kirkbride P, Prichard J, Thai PK, Carter S, Mueller JF. Profiles of illicit drug use during annual key holiday and control periods in Australia: wastewater analysis in an urban, a semi-rural and a vacation area. Addiction 2013a; 108: 556 - 65.

Lai FY, Thai PK, O´Brien J, Gartner C, Bruno R, Kele B, Ort C, Prichard J, Kirkbride P, Hall W, Carter S, Mueller JF. Using quantitative wastewater analysis to measure daily usage of conventional and emerging illicit drugs at an annual music festival. Drug Alcohol Rev 2013b; 32: 594 - 602.

Nefau T, Karolak S, Castillo L, Boireau V, Levi Y. Presence of illicit drugs and metabolites in influents and effluents of 25 sewage water treatment plants and map of drug consumption in France. Sci Total Environ 2013; 461-462: 712 – 22.

Ort C, Lawrence MG, Rieckermann J, Joss A. Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? a critical review. Environ Sci Technol 2010; 44: 6024 - 35.

Pedrouzo M, Borrull F, Pocurull E, Marcé RM. Drugs of abuse and their metabolites in waster and surface waters by liquid chromatography-tandem mass spectrometry. J Sep Sci 2011; 34: 1091 – 101.

Postigo C, López de Alda M, Barceló D. Drugs of abuse and their metabolites in the Ebro river basin: Occurrence in sewage and surface water, sewage treatment

plants removal efficiency, and collective drug usage estimation. Environ Int 2010; 36: 75 - 84.

Repice C, Dal Grande M, Maggi R, Pedrazzani R. Licit and illicit drugs in a wastewater treatment plant in Verona, Italy. Sci Total Environ 2013; 463-464: 27 - 34.

SANCO/12495/2011 (supersedes document no. SANCO/10684/2009). Method validation and quality control procedures for pesticide residues analysis in food and feed.

Thomas KV, Bijlsma L, Castiglioni S, Covaci A, Emke E, Grabic R, Hernandez F, Karolak S, Kasprzyk-Hordern B, Lindberg RH, Lopez de Alda M, Meierjohann A, Ort C, Pico Y, Quintana JB, Reid M, Rieckermann J, Terzic S, van Nuijs ALN, de Voogt P. Comparing illicit drug use in 19 European cities through sewage analysis. Sci Total Environ 2012; 432: 432 - 39.

Van der Aa M, Bijlsma L, Emke E, Dijkman E, van Nuijs ALN, van de Ven B, Hernández F, Versteegh A, de Voogt P. Risk assessment for drugs of abuse in the Dutch watercycle. Water Res 2013; 47: 1848 - 57.

van Nuijs ALN, Mougel J-F, Tarcomnicu I, Bervoets L, Blust R, Jorens PG, Neels H, Covaci A. Sewage epidemiology – a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium. Environ Int 2011a; 37: 612 - 21.

van Nuijs ALN, Castiglioni S, Tarcomnicu I, Postigo C, Lopez de Alda M, Neels H, Zuccato E, Barcelo D, Covaci A. Illicit drug consumption estimations derived from wastewater analysis: a critical review. Sci Total Environ 2011b; 409: 3564 - 77.

Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R. Estimating community drug abuse by wastewater analysis. Environ Health Perspect 2008a; 116: 1027 - 32.

Zuccato E, Castiglioni S, Bagnati R, Chiabrando C, Grassi P, Fanelli R. Illicit drugs, a novel group of environmental contaminants. Water Res 2008b; 42: 961 - 68.

Chapter 5.2.2, scientific article 9

Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry

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Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry

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HIGHLIGHTS

- We studied the presence and behavior of 24 drugs of abuse in communal sewage water.
- Analyses were performed using liquid chromatography coupled to a high resolution Orbitrap mass spectrometer.
- Samples were collected from four Dutch cities and Schiphol international airport.
- Daily variances of drug loads were demonstrated.
- Removal efficiencies were calculated for each drug and treatment plant individually.

Abstract

An extensive study on the presence of illicit drugs and pharmaceuticals with potential for abuse in sewage waters was made for the first time in the Netherlands. A total number of 24 target drugs were investigated in influent and effluent wastewater using liquid chromatography coupled to a high resolution Orbitrap mass spectrometer. This powerful analyzer has allowed not only the detection and identification of the compounds under investigation, but also their quantification at very low levels, which is highly innovative in the field of drugs of abuse. Samples were taken from five sewage treatment plants (STPs) during a whole week. The selected STPs served four cities of different size and an international airport. Daily variances of drug loads were demonstrated and removal efficiencies calculated for each drug and STP individually. Twelve target compounds were found in at least one influent or effluent, and highest concentrations were observed in influents collected from more urbanized areas. The compounds more frequently detected were amphetamine, benzoylecgonine, cocaine and THC-COOH together with the pharmaceuticals codeine, oxazepam and temazepam. Established week trends in consumption of drugs showed distinct differences between individual drugs. A slightly different occurrence pattern was observed in wastewaters from the airport. Thus, methamphetamine was only detected at Schiphol, a fact that was interpreted to be caused by consumption of this drug by travelers. Despite the fact that the Netherlands has frequently been criticized for its liberal drug policy the results from this study did not reveal higher drug consumption than found elsewhere, with the exception of cannabis.

Keywords

Illicit drugs of abuse, accurate mass, linear ion trap (LTQ FT) Orbitrap mass spectrometry, sewage water, STP removal efficiency

1. Introduction

Drugs of abuse (DOAs), either illicit or legal, are a special group of widely consumed drugs. DOAs may enter the sewage system, unaltered or as metabolites, after consumption and excretion. Data obtained from analysis of sewage water have been used to estimate consumption or to observe usage trends in communities (Zuccato et al., 2005; Van Nuijs et al., 2011). Since manufacturers, distributors and consumers are normally unknown, reliable data on the consumption of these drugs is difficult to obtain. Studies on the presence of DOA in sewage waters (influent) have provided complementary insight to the information on consumption usually obtained through enquiries and inventories. In addition, environmental loads can be calculated, and by analyzing both influent and effluent sewage waters the removal efficiency of a sewage treatment plant (STP) can be evaluated, taking into account the residence time of water in the plant (Huerta-Fontela et al., 2008; Postigo et al., 2010).

The existing methods for determination of DOAs in water are mainly based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), using triple quadrupole (QqQ) analyzers, due to its excellent characteristics for quantification at low levels, such as high sensitivity and selectivity. However, other MS analyzers are gaining interest at present. Thus, high resolution mass spectrometry (HRMS) has shown strong potential for target and non-target screening (Hernández et al., 2011a; Hogenboom et al., 2009), but it has also been proved to be an efficient tool for the simultaneous identification and quantification of DOAs at low concentrations in complex sewage water samples (Bijlsma et al., 2012). The satisfactory sensitivity in full-scan acquisition mode and high resolving power obtained by the latest generation of HRMS has facilitated its application not only to qualitative/elucidative purposes but also for quantification in the last few years (Kaufmann et al., 2011; Kellmann et al., 2009; Krauss and Hollender, 2008; Nurmi and Pellinen, 2011). An attractive feature of HRMS is that a retrospective analysis can be made from full-scan accurate mass data generated, in order to search for additional compounds not included in the first screening. This can be made without the need of additional analysis, as demonstrated previously (Bijlsma et al., 2012; Hernández et al., 2011b; Hogenboom et al., 2009).

Some exploratory studies (Hogenboom et al., 2009; Van der Aa et al., 2010) have demonstrated the presence of DOAs in Dutch waters. Nevertheless, a detailed quantitative study on wastewater influents and effluents in the Netherlands has not been performed until now. This seems interesting, since the Dutch liberal drug policy on soft drugs might lead to higher consumption of cannabis, which would result in higher concentrations in wastewater. In the present work, a recently developed and validated methodology, based on solid phase extraction (SPE) followed by LC-HRMS, has been used (Bijlsma et al., 2012). The study assesses the behavior of 24 DOAs and metabolites in five different sewage water treatment plants (STPs) from four Dutch cities and an international airport. To the best of our knowledge, this is one of the few studies reporting the monitoring of this number of DOAs during one whole week, and one of the first in this field exploiting the quantitative capabilities of the Orbitrap mass analyzer. Results contribute among others to the evaluation of the daily variances of DOAs loads at different location of the Netherlands and the removal efficiencies of STPs with highly similar treatment steps.

2. Materials and methods

2.1. Sample collection

In total, 32 influent and 32 effluent sewage water samples were collected from five STPs. The five STPs were located in the Netherlands (**Fig. S1** of the Supplementary information, SI) serving four cities (Utrecht, Eindhoven, Apeldoorn and Amsterdam) and the international airport of Amsterdam (Schiphol). The main characteristics of each STP are summarized in **Table S1** (SI). As can be seen in the table, all STPs investigated are equipped with conventional activated sludge secondary treatment and tertiary nitrogen and phosphate removal. Main differences among them refer to their water treatment capacity and the lag-time of the water in each STP.

Samples (24 h flow dependent, starting and finishing time (8:30 am to 8:30 am)) were taken during the third and fourth week of February 2010 (Wednesday to Wednesday). For Schiphol during the weekend only a composite sample reflecting an average concentration of the analyte over a 72 h time period could be collected. All 64 sewage water samples were collected in polyethylene high density bottles and stored in the

dark at 4 °C to be transported to the laboratory within 24 h maximum. Upon reception in the laboratory, samples were immediately stored in the dark at -18 °C until analysis to minimize degradation of analytes. No additional measures to delay decomposition of unstable compounds (e.g. cocaine) (Gheorghe et al., 2008) were taken.

2.2. Analytical methodology

Sample treatment and specific information on instrument operating conditions, both chromatographic and spectrometric, and on method validation can be found elsewhere (Van der Aa et al., 2010). The list of 24 target compounds investigated in this work is shown in SI. Briefly, 200 mL effluent or 100 mL influent sewage water samples were spiked with a mixed isotope labelled internal standard (ILIS) solution and extracted using SPE cartridges (Oasis HLB). After elution with methanol, extracts were evaporated and reconstructed in 500 µL of 10% methanol aqueous solution. The sample extract (20 µL) was injected directly into the LC – linear ion trap (LTQ) FT Orbitrap system. Chromatographic separation of the compounds was achieved using an XBridge C_{18} column and an optimized gradient using water:methanol, both with 0.05% formic acid. The mass spectrometer operated under data-dependent-acquisition (DDA) mode during the complete chromatographic run, in which both MS and MS^n spectra were acquired. Full-scan accurate mass spectra from 50 to 600 Da were obtained at a resolution of 30,000 FWHM. All data were acquired and processed using Xcalibur 2.1 software.

2.3. Calculation of elimination rates

Removal efficiencies were calculated by comparing effluent concentrations (C_E) from day (x + 1) with influent concentrations (C_I) from day (x), thus assuming an average residence time of 24 h (see also **Table S1**, SI). Efficiencies (*E*) were calculated as $E = (1 - (C_E/C_I))$ 100%. For each STP this resulted in six values (for Schiphol only 3), the average of which was then calculated.

3. Results and discussion

3.1. Occurrence of drugs of abuse and metabolites in Dutch wastewater

The overall frequency of detection of target compounds in the influent and effluent sewage water samples analyzed is shown in **Table 1**. The mean concentrations and the concentration ranges (in ng/L) for each compound per location are also displayed.

Twelve DOAs were found in at least one of the influents analyzed. The samples from Amsterdam showed the highest mean concentrations in influents for benzoylecgonine (2306 ng/L), cocaine (434 ng/L), the cannabis metabolite (THC-COOH, 375 ng/L) and MDMA (140 ng/L). This might be explained by the fact that Amsterdam is the largest city selected, in line with other studies where higher MDMA and cocaine consumption was related to more urbanized areas or large cities (Banta-Green et al., 2009; Van Nuijs et al., 2011). However, the highest levels of amphetamine were found in influents from Eindhoven, with concentrations ranging from 266 to 1779 ng/L, and an average of 682 ng/L.

In general, amphetamine and MDMA were at comparatively lower concentration levels than cocainics. The levels of codeine and the benzodiazepines, oxazepam and temazepam, in influents were in the same order of magnitude in all four cities, with mean concentrations ranging from 240 to 372 ng/L, 356 to 677 ng/L and 208 to 297 ng/L, respectively. Influent samples from the STP of Schiphol airport contained mean concentrations for oxazepam and temazepam slightly lower (153 and 164 ng/L, respectively), whereas codeine (536 ng/L) and cocaine (559 ng/L) were relatively higher compared to influents of municipal STPs. Two influents samples from Schiphol airport were found positive for methamphetamine (17 ng/L), while no methamphetamine was detected in any of the four Dutch cities. The reason for the occurrence of this drug might be related to international passengers travelling to or via this airport. The consumption and abuse of methamphetamine is not very popular in the Netherlands, yet much more so in East and South–East Asia (UNODC, 2010) and North–East Europe (EMCDDA, 2010).

Table 1. Overall frequency of detection[a] and mean concentration[b] of drugs of abuse in influent and effluent sewage water samples from five STPs in the Netherlands.

Compounds	FD[a] (%)		Utrecht		Eindhoven		Apeldoorn		Amsterdam		Schiphol	
	Influent (n=32)	Effluent (n=32)	Concentration (ng/L)		Concentration (ng/L)		Concentration (ng/L)		Concentration (ng/L)		Concentration (ng/L)	
			Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
Amphetamine	100	13	98[b] (41-225)	< 4	682 (266-1779)	6.9 (4.5-12)	89 (40-93)	< 4	88 (45-157)	< 4	81 (51-115)	< 4
Methamphetamine	6	-	< 15	< 5	< 15	< 5	< 15	< 5	< 15	< 5	17 (16-17)	< 5
MDMA	75	100	87 (61-132)	94 (62-131)	92 (49-142)	107 (36-222)	< 12	30 (19-49)	140 (80-241)	138 (87-190)	58 (16-85)	42 (32-55)
Cocaine	94	47	193 (142-307)	29 (10-56)	118 (99-134)	< 6	222 (87-571)	159 (103-235)	434 (203-673)	35 (35)	559 (171-957)	< 6
Benzoylecgonine	100	75	1079 (432-1560)	196 (99-351)	862 (335-1413)	21 (14-37)	409 (260-568)	102 (83-117)	2306 (1457-3701)	59 (10-155)	1472 (659-2933)	< 2
6-MAM	9	28	27 (27)	13 (12-14)	< 19	< 7	< 19	< 7	60 (47-73)	13 (7.3-30)	< 19	< 7
Methadone	-	100	< 45	38 (29-45)	< 45	8.7 (6.0-9.9)	< 45	22 (14-25)	< 45	47 (35-58)	< 45	11 (9.5-14)
Codeine	100	100	240 (73-347)	245 (121-310)	280 (119-366)	242 (97-599)	251 (113-355)	180 (89-232)	372 (230-495)	173 (134-209)	536 (336-894)	180 (145-210)
THC-COOH	88	9	183 (140-238)	13 (11-15)	131 (87-166)	< 7	91 (73-117)	< 7	375 (306-489)	22 (22)	< 33	< 7
Ketamine	22	88	< 10	8.0 (4.5-11)	17 (10-34)	44 (21-61)	< 10	7.3 (2.2-14)	< 10	12 (8.6-15)	< 10	< 2
Ritalin	-	44	< 20	5.0 (2.3-6.2)	< 20	2.1 (2.0-2.3)	< 20	< 2	< 20	< 2	< 20	< 2
Oxazepam	100	100	677 (231-915)	852 (445-994)	377 (177-494)	486 (237-586)	589 (301-882)	778 (439-908)	356 (210-430)	416 (312-476)	153 (109-245)	353 (339-367)
Temazepam	97	100	297 (99-414)	406 (208-508)	208 (92-279)	271 (133-314)	250 (209-300)	309 (159-371)	208 (139-245)	233 (174-273)	164 (121-255)	309 (290-330)
Nordazepam	41	100	9.6 (4.2-21)	9.5 (7.1-11)	4.7 (4.0-7.1)	7.1 (3.6-8.0)	< 4	7.8 (4.7-9.7)	< 4	6.1 (4.5-7.5)	< 4	8.3 (7.4-8.8)

[a]: Frequency of detection in influent and effluent sewage water samples.

[b]: Concentrations of drugs of abuse are the average of seven (one week) 24 h composite influent or effluent wastewater samples, except for Schiphol (average of three 24 h and one 72 h composite wastewater samples). Top: mean concentration; bottom: concentration range (in brackets).

It is interesting to notice that one influent sample collected from Schiphol airport and one collected in Apeldoorn showed unexpectedly high cocaine/benzoylecgonine ratios (0.85 and 2.20, respectively). Based on their molar mass relation and on the excretion rate limits as unchanged cocaine and as benzoylecgonine, cut-off values of 0.75 and 0.27 for the cocaine/benzoylecgonine ratio have been proposed (Postigo et al., 2010; Van Nuijs et al., 2009). A value above this ratio suggests that not all measured cocaine results from human consumption. Our data by far exceed these cut-off ratios, which might indicate disposal of non-consumed cocaine into the sewage water system. For Apeldoorn no clear explanation was available. However, the exceeded ratio at Schiphol airport might be related with the presence of drug traffickers, who for example due to a (sudden) surge of anxiety might unload their 'goods' into the sanitary facilities either on board the aircraft or at the airport before passing customs control. However, an extensive study, including more data and additional information (e.g. flight schedules) would be necessary to sustain this hypothesis.

Thirteen DOAs were present in at least one of the effluent sewage waters analyzed. In general, concentrations of DOAs in effluents were lower than those of influents, suggesting removal by degradation or sorption of these substances in STPs. However, for some target compounds the opposite occurred. Thus, methadone and ritalin were exclusively detected in effluents, albeit at low concentrations (maximum concentrations of 58 ng/L and 6.2 ng/L, respectively). This might be due, especially for ritalin, to the difficulties of detection and quantification of low analyte levels in influents, as a consequence of lesser pre-concentration along sample treatment step and stronger matrix ionization suppression in this type of samples.

Ketamine was mainly detected in effluents, with the exception of Eindhoven, where it was also found in influent samples (17 ng/L), however at lower concentrations than in effluent (44 ng/L). Concentrations of benzodiazepines in effluents were nearly always higher than those found in their corresponding influents. Codeine and MDMA were found occasionally at higher concentrations in the effluent sewage water. In the case of codeine, this is in agreement with the results published by others (Boleda et al., 2007).

It is also worth mentioning that the benzodiazepine diazepam was detected neither in influent nor in effluent sewage water. According to Löffler et al. (2005) diazepam undergoes fast and extensive sorption onto sediments and is highly stable in soils and during sewage water treatment. Diazepam is considered highly persistent, while oxazepam is moderately persistent in water/sediment systems. Differences in behavior of benzodiazepines are associated with differences in functional substituent groups, and – in agreement with our study – only the hydroxylated tranquilizers, oxazepam and temazepam, were reported to be present in influents and effluents (Hummel et al., 2006). In addition, oxazepam is one of the main metabolites of nordazepam and diazepam (Besse et al., 2008), and therefore it could result from oxazepam use but also from other benzodiazepines. This might also be another reason that diazepam was not detected in any sewage water, as part is excreted as oxazepam (Löffler et al., 2005). For this reason, Besse et al. (2008) suggest that oxazepam could be used as an indicator of contamination of the aquatic environment by benzodiazepines.

A detailed comparison of data obtained in this work with drug concentrations reported in the literature is problematic due to the uncertainties associated to the different steps of this type of works (e.g. sampling, stability of compounds, analytical measurements, etc.). However it seems clear that the levels of drugs and metabolites found in Dutch sewage waters are roughly of the same order of magnitude as those observed in other countries worldwide (e.g. Australia (Irvine et al., 2011), Belgium (van Nuijs et al., 2009), Italy (Castiglioni et al., 2006; Zuccato et al., 2005), Ireland and UK (Bones et al., 2007; Kasprzyk-Hordern et al., 2009), Spain (Bijlsma et al., 2009; Huerta-Fontela et al., 2008; Postigo et al., 2010) USA (Chiaia et al., 2008; Jones-Lepp et al., 2004) An exception is the relatively high concentration found for THC-COOH in influents of Dutch cities, as deduced from data shown in **Table S2** (SI). This fact might be related with the Dutch drug policy on cannabis (marijuana and hashish) usage, which is permitted for every citizen over age eighteen. In addition, the mean concentrations of MDMA in influents and effluents observed in the present study are relatively high, yet similar to those found in Barcelona and Valencia (Postigo et al., 2008), but about 10 times higher than those measured in Milan (Castiglioni et al., 2006). Morphine could not be quantified in Dutch sewage waters, surely due to the low sensitivity of the method for this compound (limits

of quantification were 360 and 125 ng/L for influents and effluents, respectively). On the contrary, significant levels (approximately 80 ng/L in influents) were found elsewhere (Boleda et al., 2007; Castiglioni et al., 2006). Nevertheless, precaution on the interpretation of the data is required, as a one-to-one comparison is difficult to make. For a correct comparison of data, various factors such as weather conditions at time of sampling, treatment, capacity and lag-times of the STPs, need to be taken into account. This would implicate a much more extensive study, organized and coordinated at the international level.

3.2. Daily variantions of drugs loads over 1 week

Four of the selected STPs serve large cities, which were considered important for studying DOAs consumption at the community level. Amsterdam is the capital city with a lot of tourists and students (~10%); Utrecht and Eindhoven are typical province towns with large student populations (>20% and 10% respectively); Apeldoorn is a town in a more 'rural' area with hardly any students (<3%). In addition, the STP from the international airport of Amsterdam (Schiphol) was selected to study consumption behavior of travelers. By plotting drug loads of influents against the days of the week, an indication can be given on the variation of DOAs consumption of each location. This approach seems appropriate, as loads of DOAs (g/d) are calculated using concentrations (ng/L), taking into account the amount of water (m^3) processed by the STP during the corresponding day (Zuccato et al., 2005). The latter is important, since the flow rate of the water stream can vary considerably (up to a factor of 3 in between days).

Daily variations of drug loads along a whole week in each STP are illustrated in **Fig. 1**. In general, loads of cocaine and its main metabolite benzoylecgonine were highest on Saturday, Sunday or Monday, suggesting a preference of cocaine consumption on weekend days. On the contrary, codeine, ketamine, benzodiazepines (e.g. oxazepam, temazepam and nordiazepam) show a continuous load throughout the week, implying a different pattern of use. The daily variances of the STP from Amsterdam, where the highest overall drug loads found in the present study are observed, also suggest increased consumption of MDMA and cannabis during weekends. These general

tendencies are consistent with reported results of monitoring studies performed in other countries over several consecutive days (Berset et al., 2010; Bijlsma et al., 2009; Huerta-Fontela et al., 2008; Terzic et al., 2010).

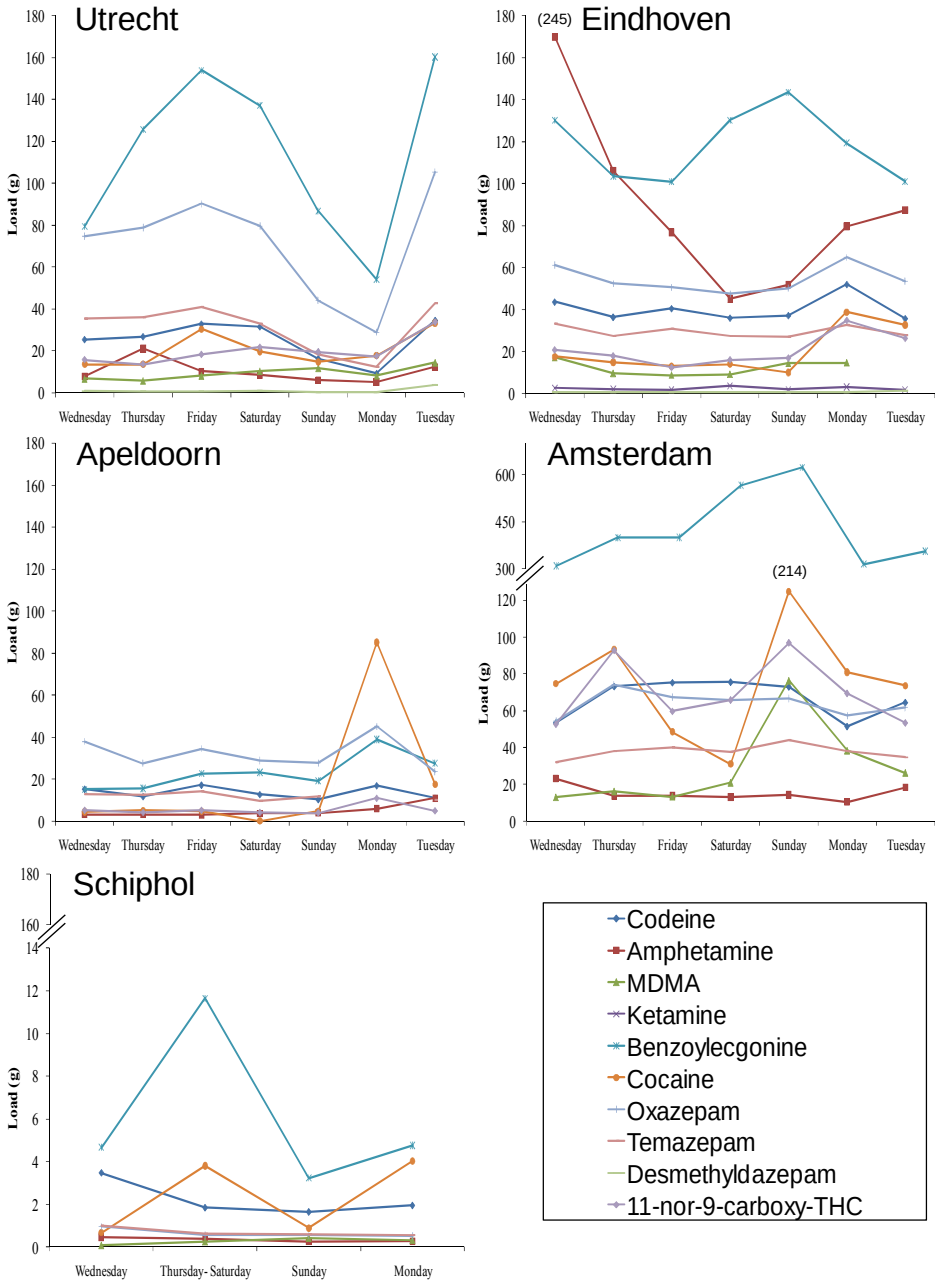

Figure 1. Daily variations of drug loads over the duration of a whole week of each STP.

At the STP of Schiphol airport, loads of DOAs were significantly lower. However, when comparing the ratio of served population (e.g. ratio Amsterdam:Schiphol, approx. 20 (**Table S1**, SI)) with loads ratio (also ~20) the consumption seemed about equal. Our results also suggest similar consumption behavior for cocaine, codeine and benzodiazepines. An interesting load pattern for cocainics was observed for the Utrecht STP, where highest loads were found on Friday and Tuesday, corresponding to the consumption of cocaine on Thursday and Monday, as the samples were collected from 8:30 am of the day before to 8:30 am of the day of sampling. The observed pattern cannot be explained by rainfall events because rainfall was invariably below 1 mm during the period Thursday to Wednesday. In addition, oxazepam shows similar daily dynamics. These results are not coherent with those observed elsewhere and an unambiguous explanation for these distinctive patterns cannot be proposed. Another unexpected consumption pattern was observed for amphetamine in Eindhoven, where relatively high loads were detected at the start of the sampling week after which the loads decreased. This may have been caused by an incidental dump or by an atypical use pattern. The actual sampling week happened to be directly after the termination of the carnival period (that ended on Tuesday) that is extensively celebrated in the city of Eindhoven. In a new European monitoring campaign started in 2011 the findings reported here will be compared.

3.3. Removal efficiency of sewage treatment plants

The analyses of sewage waters are of importance as they allow calculating removal rates for each DOA in a given STP. Good removal efficiency is essential, since Dutch STPs discharge their effluents into surface/river waters, which are important resources for drinking water production. In addition, contaminated effluents may have a potential impact on the aquatic ecosystem of the receiving water bodies.

Average removal rates, expressed as percentages, were calculated using the dissolved aqueous phase concentrations of the analytes in influent sewage waters and in their corresponding effluents. Lower levels in effluents might be a result of removal in the STP, due to microbial degradation, other transformation processes and/or sorption to the solid matter. In this work, the removal efficiencies varied significantly from 100%

elimination, when analytes were detected in the influent but were absent in the effluent, to 0% elimination, when analytes were present in influents and effluents at around the same level or when analytes were not detected in influents. "Negative" elimination rates were considered when analyte concentrations were higher in the effluent.

Estimated average removal rates for each analyte and STP are shown in **Fig. 2**. The highest removal efficiencies were observed for amphetamine (100%), methamphetamine (100%) and THC-COOH (98%), independently of the STP under study. Benzoylecgonine (90%) and cocaine (79%) also seemed to be efficiently removed in the STPs, which is in agreement with other reports (Huerta-Fontela et al., 2008; Postigo et al., 2010). However, in the STP from Apeldoorn, the elimination of cocaine and benzoylecgonine was found to be much lower (25 and 73%, respectively), and similar to the results obtained by Terzic et al. (2010). Highly variable values and even negative elimination rates of amphetamine-like drugs and THC-COOH have been reported (Boleda et al., 2007; Postigo et al., 2010). In our study, amphetamine and THC-COOH appeared to be efficiently removed in all STPs, as well as methamphetamine in the only STP where it was detected (Schiphol). Loganathan et al., (2009) reported less than 100% removal efficiency of methamphetamine, but this difference could be accounted for due to differences in STP treatment processes and the amount of methamphetamine coming into the STP. MDMA showed highest variability, but in general with poor elimination, ranging from −12 to 26%. High differences and lower removal efficiencies were also found among opiates, with average values of 23 and 37% for 6-MAM and codeine, respectively. Kasprzyk-Hordern et al. (2009) found similar results and related a more effective removal of amphetamine, cocaine and benzoylecgonine to activated sludge treatment, whereas a lower removal efficiency (42%) was found for codeine using the same technology.

As mentioned earlier, benzodiazepines were found nearly always at higher concentrations in effluents than in influents, resulting in "negative" removal rates (**Fig. 2**). Accordingly, average removal efficiencies for oxazepam, temazepam and nordazepam were −46, −38 and −18%, respectively. This might be related to the

cleavage of the conjugated molecules in influent sewage water, as demonstrated for estrogens (D'Ascenzo et al., 2003; Ternes et al., 1999). Deconjugation of glucoronides that can occur during sewage water treatment, can also play an important role for other compounds, such as codeine and benzodiazepines, involving deconjugation of codeine-6-glucuronide, temazepam- and oxazepam-glucuronide during the treatment process (Boleda et al., 2007).

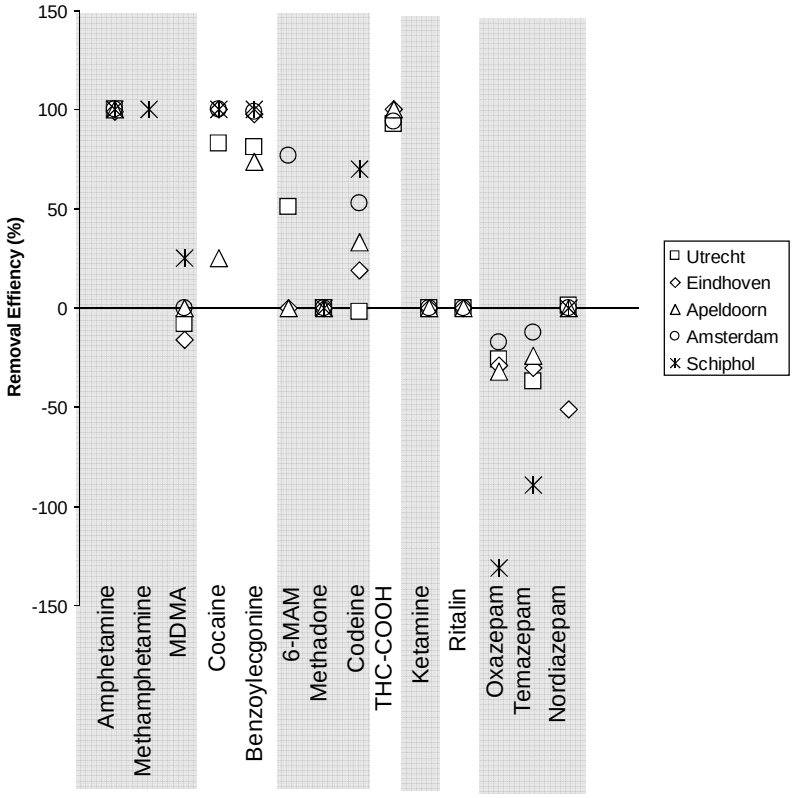

Figure 2. Average removal efficiencies of drugs of abuse and metabolites in the investigated STPs.

The application of Principal Component Analysis (PCA) using Statgraphics 7.0 helped us to evaluate the removal efficiency of the compounds investigated for each STP. Data were converted to Log (concentrations + 1) to correct for the dependence between arithmetic means and standard deviations. Homoscedasticity of variances was tested by means of Barlett's test ($P < 0.05$). The plot of the two first components explains 92% of the total variance (**Fig. S2**, SI). The distance among variables, clearly separated by

component 2, could be interpreted as the removal efficiency of the contaminants within each STP. The poorest removal efficiencies were observed for the treatment plants from Utrecht (u) and Apeldoorn (a). This might be related to their lower lag-times, i.e. the time it takes water to enter and leave the STP. Low lag-time was also related to poor elimination in the study of Postigo et al. (2010).

The analyses of both influents and effluents also allowed us to estimate the lag-time (hydraulic retention time) of the plant, demonstrating another applied issue of analyzing chemicals in sewage waters. Lag-times can be estimated by using a marker compound, which is present in both influent and effluent sewage water. In our case, we used benzoylecgonine as a marker. Assuming that the lag-time and removal efficiency are constant, the week profiles of benzoylecgonine in influents and effluents be superimposed; the observed shift (visible as time difference between influent and effluent curves, notably minimums and maximums) then corresponds to the lag-time of the treatment plant. As an example, **Fig. 3** shows the estimated lag-time for Apeldoorn (13 h) and Amsterdam (24 h), which are comparable with the data put at our disposal by the STP managers (10 and 24 h, respectively).

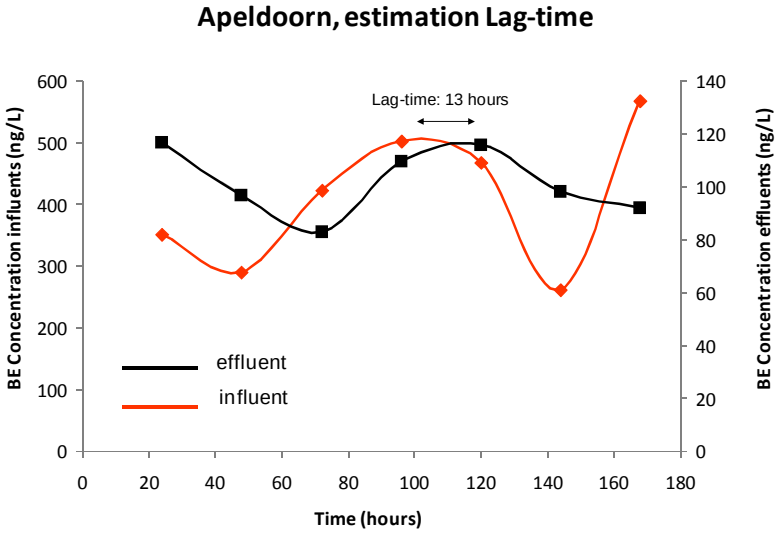

Figure 3. Time plots vs. concentration of benzoylecgonine (BE) demonstrating estimation of lag-time for two STPs: ***Apeldoorn (top)*** and Amsterdam (bottom).

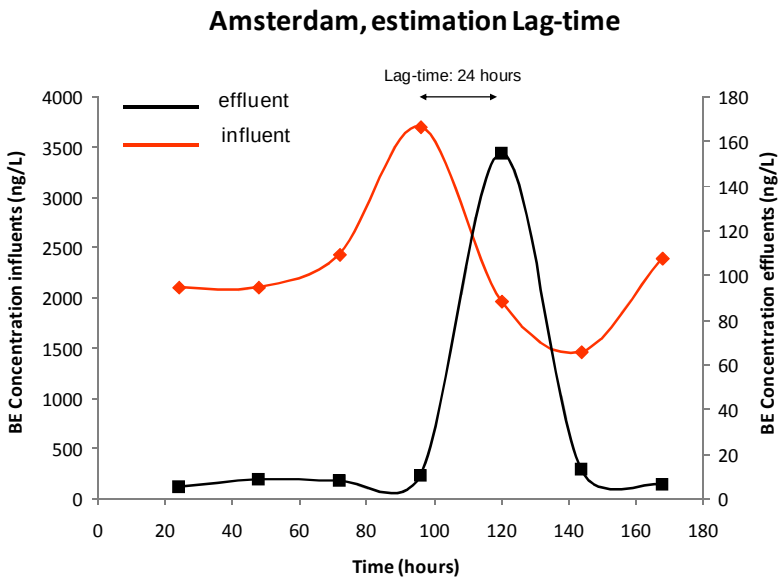

Figure 3. Time plots vs. concentration of benzoylecgonine (BE) demonstrating estimation of lag-time for two STPs: Apeldoorn (top) and ***Amsterdam (bottom)***.

4. Conclusions

Advanced analytical methodology based on the use of LC-LTQ FT Orbitrap MS has been applied in this work for the simultaneous quantification and confirmation of 24 target drugs of abuse in sewage water. The results of an extensive week monitoring contributed to a better insight on drugs of abuse in the Netherlands and their presence in Dutch influent and effluent sewage water. Data of this work allowed evaluating removal efficiencies of the selected STPs, which were generally satisfactory except for benzodiazepines and MDMA. Week monitoring of analytes in both influents and effluents also allow estimating lag-times of each STP. The inclusion of the STP of the international airport of Schiphol, the size of which is equivalent to a small town but presumably reflecting differences in drug consumption compared to common townships, is innovative in this type of work and, to the best of our knowledge, has not been previously reported in the literature.

Acknowledgements

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Supplementary data

In this section, a list of compounds investigated, a table with the main characteristics of the STPs under study (Table S1) and a table comparing concentrations of THC-COOH in influent sewage water (Table S2) are included. Furthermore, two figures, one including the locations of the investigated STPs in the Netherlands (Figure S1), and another showing a PCA plot for removal efficiencies of different STPs (Figure S2), are added to provide supplementary information to the written text.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2012.05.110 and in this chapter after section "References".

References

Banta-Green, C., Field, J., Chiaia, A.C., Sudakin, D.L., Power, L., de Montigny, L., 2009. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater. Addiction 104, 1874 – 1880.

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

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

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

Bijlsma, L., Emke, E., Hernández, F., de Voogt, P., 2012. The performance of Orbitrap analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water. Anal. Bioanal. Chem. Submitted.

Bones, J., Thomas, K.V., Brett, P., 2007. Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals. J. Environ. Monitor 9, 701 – 707.

Boleda, M.R., Galceran, M.T., Ventura, F., 2007. Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry. J. Chromatogr. A 1175, 38 - 48.

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

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

D'Ascenzo, G., Di Corcia, A., Gentili, A., Mancini, R., Mastropasqua, R., Nazzari, M., Samperi, R., 2003. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. Sci. Total Environ. 302, 199 - 209.

EMCDDA, European Monitoring Centre for Drugs and Drug Addiction 2010. The state of the drugs problem in Europe. EMCDDA Annual Report, Lisbon.

Gheorghe, A., van Nuijs, A., Pecceu, B., Bervoets, L., Jorens, P. G., Blust, R., Neels, H., Covaci, A., (2008). Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry. Anal. Bioanal. Chem. 391, 1309-1319

Hernández, F., Bijlsma, L., Sancho, J.V., Díaz, R., Ibáñez, M., 2011a. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. Anal. Chim. Acta 684, 96 - 106.

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

Hogenboom, A.C., van Leerdam, J.A., de Voogt, P., 2009. Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography - hybrid linear ion trap Orbitrap mass spectrometry. J. Chromatogr. A 1216, 510 – 519.

Huerta-Fontela, M., Galceran, M.T., Martin-Alonso, J., Ventura, F., 2008. Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain. Sci. Total Environ. 397, 31 - 40.

Hummel, D., Löffler, D., Fink, G., Ternes, T.A., 2006. Simultaneous determination of psychoactive drugs and their metabolites in aqueous matrices by liquid chromatography mass spectrometry. Environ. Sci. Technol. 40, 7321 - 7328.

Irvine, R.J., Kostakis, C., Felgate, P.D., Jaehne, E.J., Chen, C., White, J.M., 2011. Population drug use in Australia: a wastewater analysis. Forensic Sci. Int. 210, 69-73.

Jones-Lepp, T.L., Alvarez, D.A., Petty, J.D., Huckins, J.N., 2004. Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents. Arch. Environ. Con. Tox. 47, 427- 439.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters. Water Res. 43, 363 - 380.

Kaufmann, A., Butcher, P., Maden, K., Walker, S., Widmer, M., 2011. Quantitative and confirmative performance of liquid chromatography coupled to high-resolution mass spectrometry compared to tandem mass spectrometry. Rapid Commun. Mass Sp. 25, 979 – 992.

Kellmann, M., Muenster, H., Zomer, P., Mol, H., 2009. Full scan MS in comprehensive qualitative and quantitative residue analysis in food and feed matrices: how much resolving power is required? J. Am. Soc. Mass Spectr. 20, 1464 – 1476.

Krauss, M., Hollender, J., 2008. Analysis of nitrosamines in wastewater: exploring the trace level quantification capabilities of a hybrid linear ion trap/orbitrap mass spectrometer. Anal. Chem. 80, 834 - 842.

Löffler, D., Römbke, J., Meller, M., Ternes, T.A., 2005. Environmental fate of pharmaceuticals in water/sediment systems. Environ. Sci. Technol. 39, 5209 - 5218.

Loganathan, B., Phillips, M., Mowery, H., Jones-Lepp, T.L., 2009. Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant. Chemosphere 75, 70-77.

Nurmi, J., Pellinen, J., 2011. Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry. J. Chromatogr. A 1218, 6712 – 6719.

Postigo, C., Lopez de Alda, M.J., Barceló, D., 2008. Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography-electrospray-tandem mass spectrometry. Anal. Chem. 80, 3123 – 3134.

Postigo, C., López de Alda, M., Barceló, D., 2010. Drugs of abuse and their metabolites in the Ebro river basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation. Environ. Int. 36, 75 – 84.

Ternes, T.A., Kreckel, P., Mueller, J., 1999. Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic bath experiments with activated sludge. Sci. Total Environ. 225, 91 – 99.

Terzic, S., Senta, I., Ahel, M., 2010. Illicit drugs in wastewater of the city of Zagreb (Croatia) – Estimation of drug abuse in a transition country. Environ. Pollut. 158, 2686 - 2693.

UNODC (United Nations Office on Drugs and Crime). World Drug Report, 2010. Available from URL: http://www.unodc.org/unodc/en/data-and-analysis/WDR-2010.html (accessed January 2012)

Van der Aa, N.G.F.M., Dijkman, E., Bijlsma, L., Emke, E., van de Vem, B.M., van Nuijs, A.L.N., de Voogt, P., 2010. Drugs of abuse in Dutch drinking water, surface water and wastewater. RIVM report 703719064, Bilthoven, 1-90. Available from URL: www.rivm.nl (accessed January 2012).

Van Nuijs, A.L.N., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P.G., Bervoets, L., Blust, R., Meulemans, H., Neels, H., Covaci, A., 2009. Can cocaine use be

evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium. Addiction 104, 734 – 741.

Van Nuijs, A.L.N., Castiglioni, S., Tarcomnicu, I., Postigo, C., Lopez de Alba, M., Neels, H., Zuccato, E., Barceló, D., Covaci, A., 2011. Illicit drug consumption estimations derived from wastewater analysis: a critical review. Sci. Total Environ. 409, 3564 – 3577.

Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S., Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. Environ. Health 4:14, Available from URL: http://www.ehjournal.net/content/4/1/14 (accessed January 2012).

Supplementary data

Drugs of abuse and metabolites

Drugs of abuse and metabolites studied were: amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylene-dioxymethamphetamine (MDMA, or ecstasy), 3,4-methylenedioxyethylamphetamine (MDEA), cocaine, benzoylecgonine, heroin, morphine, 6-monoacetyl morphine (6-MAM), methadone, codeine, Δ-9-tetrahydrocannabinol (THC), 11-nor-9-carboxy-Δ-9-tetrahydrocannabinol (THC-COOH), 11-hydroxy-Δ-9-tetrahydrocannabinol (OH-THC), ketamine, ritalin, oxazepam, diazepam, temazepam, nordazepam, desalk-flurazepam, meta-CPP, and fentanyl. Standards were obtained from Lipomed AG (Arlesheim, Switzerland) as solutions in methanol, ethanol or acetonitrile at a concentration of 1 g/L.

Table S1: Characteristics of the investigated STPs.

Id	Location	Population Served	Origin[a]	Average influent flowrate (m^3/d)	LT[b] (h)	Treatment[c]
1	Utrecht	529.000	U	110.306	17	Physical, biological, removal (N, P)
2	Eindhoven	544.030	U	169.809	24	Physical, biological, removal (N, P)
3	Apeldoorn	351.500	U + I	62.122	10	Physical, biological, removal (N, P)
4	Amsterdam	913.435	U	191.041	24	Physical, biological, removal (N, P)
5	Schiphol	40.000	U + I[d]	4173	27	Physical, biological, removal (N, P)

[a] urban (U); industrial (I)

[b] The lag-time (LT) was calculated by using the total volume of all the separate traps in the STP and the average flow rate during the sampling campaign

[c] Primary step: physical treatment (incl. grit removal), secondary step: biological treatment (activated sludge), tertiary step: N, P removal.

[d] 50% originates from passengers and companies located on Amsterdam Airport Schiphol, 25% originates from aircrafts and catering, and 25% related industry around Schiphol.

Table S2: Comparison of concentrations of THC-COOH in influent sewage water.

	THC-COOH Concentration range (ng/L)
The Netherlands (this work)	
Utrecht	140 - 238
Eindhoven	87 - 166
Apeldoorn	73 - 117
Amsterdam	306 - 489
Italy (Castiglioni et al. 2006)	62.7 - 91.2
Spain (Boleda et al. 2007)	< 12.5 - 96.2
Spain (Postigo et al. 2010)	10.6 - 21.7
Switzerland (Berset et al. 2010)	< 100
Croatia (Terzic et al. 2010)	21 - 128

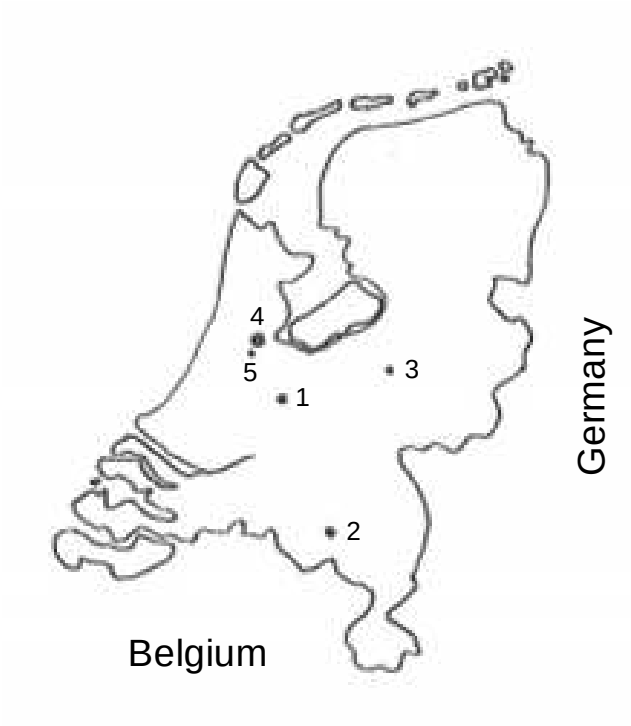


Figure S1: Locations of the investigated STPs in the Netherlands. (1) Utrecht, (2) Eindhoven, (3) Apeldoorn, (4) Amsterdam and (5) the international airport of Amsterdam (Schiphol).

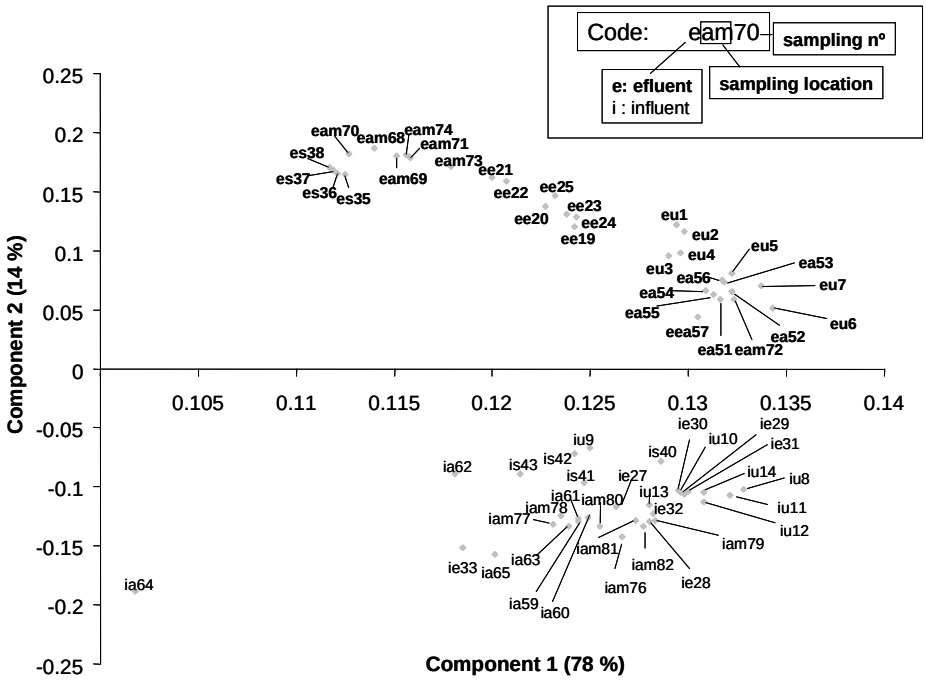
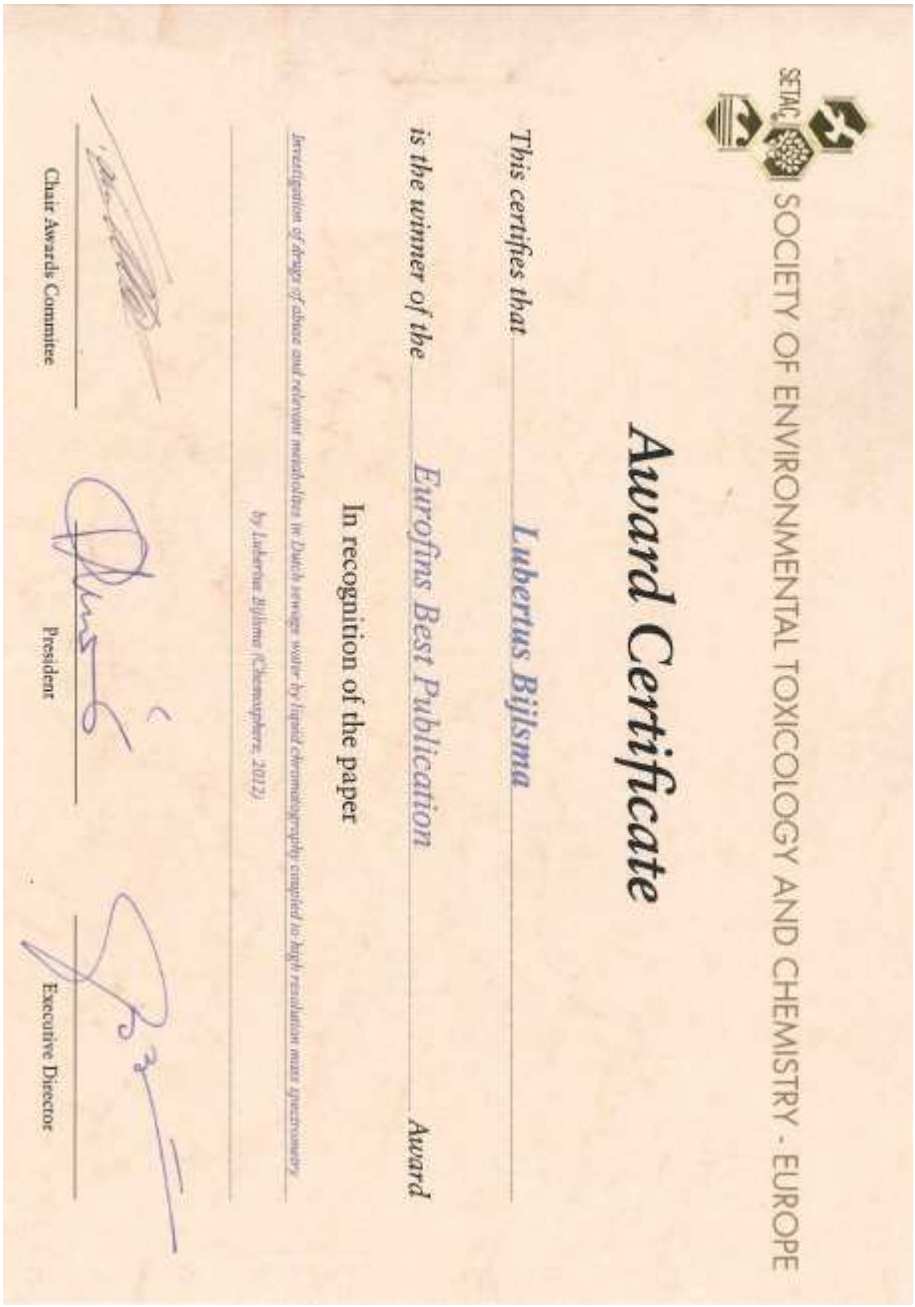


Figure S2: PCA plot of DOA concentrations determined in influent and effluent sewage water from different sample locations. (Utrecht (u); Eindhoven (e); Apeldoorn (a); Amsterdam (am); Schiphol (s)).

Eurofins Best Publication award 2012, given by the Society of Environmental Toxicology and Chemistry (SETAC) – Europe.



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Chapter 5.2.3, scientific article 10

Risk assessment for drugs of abuse in the Dutch watercylce

Monique van der Aa, Lubertus Bijlsma, Erik Emke, Ellen Dijkman, Alexander L.N. van Nuijs, Bianca van de Ven, Félix Hernández, Ans Versteegh, Pim de Voogt

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Risk assessment for drugs of abuse in the Dutch watercycle

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Graphical Abstract

Highlights

- The Dutch water cycle was screened for the presence of 34 drugs of abuse (DOA)
- Samples were analysed by four different laboratories using fully in-house validated methods
- DOA were determined in influents (17) and effluents (22), surface water (9), and raw water (3)
- Neither environmental effects nor human health risks are expected for the detected DOA

Abstract

A screening campaign of drugs of abuse (DOA) and their relevant metabolites in the aqueous environment was performed in the Netherlands. The presence of DOA, together with the potential risks for the environment and the possible human exposure to these compounds through consumption of drinking water was investigated. Sewage water (influent and effluent), surface water of the rivers Rhine and Meuse, and drinking water (raw and finished) were analysed by four different laboratories using fully in-house validated methods for a total number of 34 DOA and metabolites. In this way, data reported for several compounds could also be confirmed by other laboratories, giving extra confidence to the results obtained in this study. In total 17 and 22 DOA were detected and quantified in influent and effluent sewage samples, respectively. The tranquilizers oxazepam and temazepam, and cocaine and its metabolite benzoylecgonine were found in high concentrations in sewage water. Nine compounds were possibly not efficiently removed during treatment and were detected in surface waters. The results indicated that substantial fractions of the total load of DOA and metabolites in the rivers Rhine and Meuse enter the Netherlands from abroad. For some compounds, loads appear to increase going downstream, which is caused by a contribution from Dutch sewage water effluents. As far as data are available, no environmental effects are expected of the measured DOA in surface waters.

In raw water, three DOA were detected, whereas in only one finished drinking water out of the 17 tested, benzoylecgonine was identified, albeit at a concentration below the limit of quantification (<1 ng/L). Concentrations were well below the general signal value of 1 µg/L, which is specified for organic compounds of anthropogenic origin in the Dutch Drinking Water Act.

Keywords

Drugs of abuse, sewage water, surface water, drinking water, environmental risk characterization

1. Introduction

Drugs of abuse (DOA) and their metabolites have recently been recognised as a novel group of environmental contaminants (Zuccato et al., 2008a). Owing to the increased sensitivity of analytical methods and the high level of world-wide consumption of DOA, they are among the growing number of emerging compounds that are detected at trace concentrations in the aqueous environment, including sewage water and surface waters.

DOA refers to both illegal drugs and misused prescription drugs, such as tranquilizers. They have received special attention recently since a novel approach allowed to study DOA consumption patterns of a population through sewage water analysis (Daughton, 2001; Zuccato et al., 2008b; van Nuijs et al., 2010; Thomas et al., 2012). Following consumption and excretion, some DOA and their metabolites are continuously released into the aquatic environment due to their insufficient elimination in sewage treatment plants (STPs) (Huerta-Fontela et al., 2008; Kasprzyk-Hordern et al., 2009; van Nuijs et al., 2009a; Postigo et al., 2010). Recent studies have shown the presence of DOA and their metabolites in STP effluents and river water in Australia (Irvine et al., 2011), Europe (Boleda et al., 2009; van Nuijs et al., 2009a; Postigo et al., 2010; Baker and Kasprzyk-Hordern, 2011; Hernandez et al., 2011) and North America (Jones-Lepp et al., 2004; Bartelt-Hunt et al., 2009).

Although the reported concentrations in surface waters are in general low, possible toxicological effects on animals, plants and humans may occur as a result of their presence in the aquatic environment. Especially, long-term effects on organisms and the effects of combined exposure to multiple compounds are of potential concern. However, so far, little ecotoxicological information for DOA is available and a well-founded scientific risk assessment is not yet possible. Although some information is available on DOA removal and transformation products formed during (drinking) water treatment processes (Huerta-Fontela et al., 2008), much more research is required for a better knowledge and understanding of these processes. In the Netherlands, where approximately 40% of the drinking water is produced from surface water, little is known about the occurrence of DOA and their metabolites in the Dutch water cycle.

Exploratory studies conducted in the period 2007–2010 have revealed the presence of benzoylecgonine, methadone, codeine and three tranquilizers (nordazepam, temazepam and oxazepam) in Dutch surface waters and sewage effluents (de Voogt et al., 2011; Hogenboom et al., 2009). The results from this study implied a clear need for a more detailed monitoring campaign in the Netherlands.

This work presents the results of a large monitoring exercise on the occurrence of DOA and metabolites in the Dutch watercycle. To the best of our knowledge, this study is one of the largest of this kind in Europe, both in terms of number of analytes investigated and types of water studied. In addition, samples were individually analysed by four different laboratories, using their own validated analytical methodology. Five DOA were determined by all four laboratories and additional seven by at least two laboratories. The fact that three DOA (amphetamine, MDMA and benzoylecgonine) were found in several water samples by all laboratories allowed the performance of an interlaboratory exercise.

Beforehand, a selection of compounds was made, applying the following criteria: the results of the aforementioned preliminary inventory studies; international occurrence data on DOA and metabolites in the aqueous environment (Baker and Kasprzyk-Hordern, 2011; Bartelt-Hunt et al., 2009; Boleda et al., 2009; Hernandez et al., 2011; Irvine et al., 2011; Jones-Lepp et al., 2004; Postigo et al., 2010; van Nuijs et al., 2009a); the estimated DOA consumption in the Netherlands, which was based on criteria such as (il)legal import volumes and anonymous surveys (van Laar et al., 2007), the availability of reference standards and internal isotope-labelled standards, and the scope of the methods applied by the different laboratories participating.

The main objectives pursued within this study were (1) to evaluate the occurrence of DOA and metabolites in the Dutch watercycle (sewage influents and effluents, surface water and drinking water); (2) to perform an ecotoxicological risk assessment of the levels of DOA observed in surface waters.

2. Methods and materials

2.1. Sampling sites and sample collection

The sampling campaign in this study was performed by the Dutch National Institute for Public Health and the Environment (RIVM). All water samples were analysed by three laboratories: RIVM, KWR Watercycle Research Institute and University Jaume I (UJI). In addition, sewage water samples from four STPs (Utrecht, Apeldoorn, Amsterdam, Eindhoven) were also analysed by the University of Antwerp (UA).

Figure S1 of the Supplementary Information (SI) presents an overview of the sampling locations. Samples were collected from 65 sites and corresponded to three different types of water:

(1) Surface water: samples were collected at all nine surface water intake points for drinking water production in the Netherlands. Eight of these locations were part of the Meuse and Rhine river basins, and one was part of the Ems river basin. In addition, samples were taken at five locations along the rivers Rhine and Meuse.
(2) Raw water and finished drinking water: samples were taken at ten production sites where drinking water is produced from surface water and another seven drinking water production sites where drinking water is produced from river bank filtration. Raw water refers to the source water that enters the drinking water production facility. At some production sites this raw water has undergone pre-treatment, e.g., direct filtration, subsoil passage in the dune areas or storage in a reservoir, before it enters the drinking water treatment plant. Finished drinking water refers to water that is distributed as tap water. Drinking water treatment mostly consists of a combination of coagulation/flocculation and filtration/flotation, UV/H_2O_2 treatment or ozonation followed by activated carbon filtration.
(3) Sewage water: influent and effluent water samples were collected from eight STPs. The size of these conventional biological treatment facilities varies from 37,000 to 1 million equivalent-inhabitants.

Samples were collected in 2009 between October 4th and November 1st. At each sampling location for surface and drinking water, grab samples were taken. At the drinking water production sites, both raw water and finished drinking water were sampled on the same day, without accounting for lag-time. At the STPs, 24-h flow dependent samples from influent and effluent were collected on the same weekend day, without accounting for lag-time within the STP. All samples were collected in amber glass bottles, and transported and stored in the dark at 4 °C.

2.2. Selection of analytes

A total of 34 DOA and metabolites belonging to 6 different chemical classes were selected. The list of compounds, and isotopically labelled internal standards (ILIS) used for matrix effects correction and quantification, by the four participating laboratories, and details on preparation and storage of standard solutions are given in SI and **Table S1**.

2.3. Analytical methods

Table 1 presents an overview of the main characteristics of the analytical methods used by the four laboratories that participated in this study.

Sample clean-up and preconcentration was achieved by off-line solid-phase extraction (SPE). Analyses of the final sample extracts were performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). All instruments employed electrospray ionization (ESI) operating in positive mode. The applied mass spectrometric techniques were triple quadrupole mass analyzers (QqQ), except for KWR that used high-resolution mass spectrometry (LTQ FT Orbitrap). Further details on the analytical procedures and instrument parameters can be found elsewhere (UJI (Bijlsma et al., 2009), KWR (De Voogt et al., 2011), UA (van Nuijs et al., 2009b)), except for RIVM which is described in *Supplementary Information*.

Table 1: Summary of the analytical methods used by the four laboratories.

	Sample volume (mL)	Pre- treatment	pH adjustment	SPE column	Type of analytical LC column	Final volume extract (µL)	Injection volume (µL)	Amount of sample analysed (mL)	Conc. factors
RIVM	100	none	No	Oasis HLB (6 cc, 200 mg)	C_{18}	400	25	6.25	250
KWR	900	filtration	pH 7	Oasis HLB (6 cc, 150 mg)	C_{18}	500	20	36	1800
UJI	50	centrifugation	pH 2	Oasis MCX (6 cc, 150 mg)	C_{18}	1000	20	1	50
UA	50	filtration	pH 2	Oasis MCX (3 cc, 60 mg)	HILIC	200	5	1.25	250

2.4. Quality assurance

The analytical methods used in the present study were validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision (De Voogt et al., 2011; Bijlsma et al., 2009; van Nuijs et al., 2009b). ILIS were used to compensate for matrix effects (Hernández et al., 2005; Vanderford and Snyder, 2006). The identity of each of the investigated analytes in samples of wastewater, surface water and drinking water was confirmed by fulfilling relative retention time criteria and mass spectrometric identification criteria (Commission Decision, 2002/657/EC). An overview of the LOQs of the different methods applied can be found in **Table S2**.

2.5. Environmental risk characterization

Environmental risk characterization for substances is usually performed by calculating a Risk Characterization Ratio (RCR), which is a PEC/PNEC or MEC/PNEC ratio, in which PNEC (Predicted No Effect Concentration) is an estimate for the highest concentration of substance not affecting aquatic ecosystems, and PEC or MEC is the Predicted or Measured Environmental Concentration in the aquatic environment. If the RCR is < 1, no potential risk to the aquatic environment is expected. A literature search was carried out to obtain PNECs for the DOA detected in surface waters. In 2007, the Norwegian Pollution Control Authority collected PNECs of pharmaceuticals, narcotics, and personal care products. For compounds where no effect data were available, they used Quantitative Structure-Activity Relationship (QSAR) or Ecological Structure Activity Relationships (ECOSAR) models to estimate the potential effects of each compound ($PNEC_{ECOSAR}$) (Grung et al., 2007).

3. Results and discussion

3.1. Comparative analysis between laboratories

As mentioned above, all water samples were analysed by three laboratories: RIVM, KWR and UJI. Some of the STP wastewater samples were also analysed by the UA. To the best of our knowledge, this study is unique with respect to the number of different laboratories and methodologies involved in analysing the same water samples. From the total of 34 DOA and metabolites that were analysed in this monitoring campaign,

12 compounds were analysed by two or more laboratories. Three of these DOA (amphetamine, MDMA, and benzoylecgonine) were detected in sewage water by all four laboratories. This allowed us to perform an extra validation of the methodology applied, a relevant aspect taking into account the analytical difficulties associated with these complex sample matrices. So, in addition to the criteria applied by each laboratory to assure quality, the deviations between the results reported by the participants were used to prove the reliability of the analytical methods applied.

Table S3 shows comparative data obtained for the analysis of these three DOA in ten sewage waters (analysed by four laboratories) and six surface waters (analysed by three laboratories). Relative standard deviations (RSD) and overall average concentrations for the 16 samples analysed are shown in the Supplementary Information. In general, the overall (among laboratories) RSD was between 7 and 26%, with the exception of the RSD for benzoylecgonine in two STP effluent samples (RSD = 38%). The fact that samples were analysed using different methodologies and that reported concentrations were comparable, renders high confidence to the results obtained.

3.2. Drugs of abuse and metabolites in the Dutch water cycle

An overview of the monitoring results of DOA in the Dutch water cycle is presented in **Table 2**. The average ± standard deviation (SD), range and median of the quantified levels illustrate the dispersion and variation of the obtained results. Out of the total number of 34 DOA and metabolites analysed, 24 compounds were detected and quantified in sewage water, 9 in surface water, 3 in raw and none in finished drinking water. The presence of benzoylecgonine was confirmed in one finished drinking water sample, but at a concentration below the LOQ for this analyte (1 ng/L). It must be considered that only a single, 24-h composite sample from the effluents was collected to estimate loads of DOA discharged from the STP, and that these samples were collected during the weekend. It is well-known that concentrations of some DOA are higher during the weekend compared to weekdays (Thomas et al., 2012). So the average loads might be different from the loads calculated in this paper. Therefore, this might be seen as the worst-case scenario because of the higher concentrations found

in sewage water. Similarly, loads of DOA into the rivers were calculated using only a single grab sample per location, which is a limitation when comparing the loads from different locations and countries. However, the data presented in this work provides a valuable indication of the importance of STP discharges of DOA into the environment. The daily and seasonal variations of discharge loads were not an objective of the present study and should be evaluated in a new set of experiments.

3.2.1. Occurrence in sewage water

In STP influents, 17 compounds could be quantified, while for effluents 22 compounds showed concentrations > LOQ (**Table 2**). The compounds found in the STP influents were also detected in the STP effluents, except for THC-COOH and cocaethylene, whereas MDA, diazepam, nordazepam, fentanyl, ketamine, methcathinone and ritalin were solely found in effluents. Deconjugation within the STP, transformation of compounds (e.g. in the case of benzodiazepines), the higher LOQs in influent samples compared with effluents, or a combination of these processes might explain the exclusive presence and/or higher concentrations found in effluent compared to influent samples (Bones et al., 2007; Kvanli et al., 2008). To define which process occurs for which compound is beyond the scope of this study and should be a focus of completely new experiments. Moreover, conclusions about removal efficiencies of the STPs cannot be drawn based on this research, since STP influents and effluents were collected on the same day and as a result lag-times were not taken into account. In a later study, removal efficiencies and daily variations were investigated in an extensive one week monitoring of 24 DOA and metabolites in Dutch influent and effluent sewage water (Bijlsma et al., 2012). Occurrence of DOA monitored by both studies is in a good agreement. From the 18 common compounds included in both studies, 14 compounds were detected in influents and/or effluents in both cases. The only exceptions were MDA, diazepam, morphine and fentanyl that were not found in any sewage water sample analysed by Bijlsma et al. (2012).

Table 2: Occurrence of DOA and metabolites in Dutch waters (levels quantified (average ± standard deviation (SD), range and median))

	Influent sewage water				Effluent sewage water				Surface water				Raw drinking- / process water			
		Concentration (ng/L)				Concentration (ng/L)				Concentration (ng/L)				Concentration (ng/L)		
	FD[a]	Average ± SD	Range	Median	FD[a]	Average ± SD	Range	Median	FD[a]	Average ± SD	Range	Median	FD[a]	Average ± SD	Range	Median
Amphetamine	8/8	334 ± 179	107 - 581	310	1/8	15										
Metamphetamine	2/8	151 ± 180	24 - 278	151	4/8	37 ± 20	13 - 62	33	1/14	1						
MDA					1/8	22										
MDMA	8/8	109 ± 51	42 - 207	102	8/8	126 ± 174	17 - 537	56	4/14	2 ± 1	1 - 2	2				
Diazepam					5/8	4 ± 1	2 - 5	3								
Nordazepam					5/8	19 ± 7	13 - 31	18								
Oxazepam	8/8	1167 ± 445	602 - 2020	1105	8/8	1122 ± 375	713 - 1746	959	12/14	29 ± 22	6 - 68	25	7/17	8 ± 5	3 - 13	8
Temazepam	8/8	427 ± 179	255 - 813	411	8/8	568 ± 198	389 - 1016	554	12/14	12 ± 12	3 - 32	6	7/17	4 ± 4	1 - 10	3
THC-COOH	7/8	424 ± 137	289 - 678	378												
Cocaine	8/8	438 ± 245	135 - 904	363	6/8	4 ± 3	1 - 11	3	2/14	2 ± 1	1 - 3	2				
Benzoylecgonine	8/8	1703 ± 870	570 - 2907	1463	8/8	26 ± 25	7 - 84	20	10/14	5 ± 4	1 - 16	3	5/17	2 ± 1	1 - 3	1
Cocaethylene	7/8	27 ± 19	8 - 62	19												
Norbenzoylecgonine	6/8	36 ± 16	18 - 60	38	4/8	4 ± 1	3 - 5	4								
Norcocaine	6/8	20 ± 10	10 - 39	17	1/8	4										
Ecgonine methylester	4/4[b]	207 ± 97	84 - 312	216	3/4[b]	41 ± 2	3 - 6	3								
6-MAM	1/8	3			2/8	5 ± 2	3 - 6	5								
Morphine	8/8	665 ± 418	300 - 1464	517	7/8	31 ± 22	7 - 68	20	1/14	7						
Codeine	8/8	580 ± 230	300 - 975	526	8/8	192 ± 88	110 - 378	168	7/14	7 ± 8	1 - 23	4				
Methadone	4/8	37 ± 20	16 - 64	34	8/8	29 ± 19	6 - 56	22	3/14	2 ± 1	1 - 2	2				
EDDP	4/4[b]	84 ± 41	36 - 135	82	4/4[b]	73 ± 43	25 - 128	67								
Fentanyl					1/8	8										
Ketamine					6/8	16 ± 12	2 - 28	10								
Methcathinone					1/8	4										
Ritalin					6/8	5 ± 3	2 - 9	6								

[a]: Frequency of determination

[b]: Analyzed by UA (STPs: Utrecht, Apeldoorn, Amsterdam, Eindhoven)

In addition, nordazepam, ketamine and ritalin were mainly found in effluents, which is in correspondence with the results of the present work. A preliminary conclusion that can be drawn from the present study is that 22 out of 34 DOA were not completely removed during sewage water treatment. As a consequence, substantial loads of DOA and metabolites may enter receiving surface waters through STP effluents.

Fig. 1 shows the calculated loads of DOA discharged from the eight Dutch STP effluents collected during a weekend day. The Amsterdam STP shows highest loads towards surface water, up to 105 g/day of oxazepam. This can be related to the highest Inhabitant Equivalent (I.E.) for this STP, and also to the higher consumption of DOA that is expected in more urbanized areas or large cities (van Nuijs et al., 2009a; Banta-Green et al., 2009). Hence, if removal efficiencies (%) are of the same order of magnitude for all STPs, higher discharges can be expected when higher I.E.s are involved. However there are some exceptions, indicating that other factors also play a role (e.g. consumption of certain DOA can be regionally and temporally dependent). A noticeable discharge is shown for MDMA in Amsterdam (up to 80 g/day, 10 fold more than any of the other STPs). An estimation of the discharges expressed per inhabitant also indicated highest loads of MDMA for Amsterdam (data not shown). In general, discharge values of DOA expressed per inhabitant correspond when comparing the different cities. A possible explanation for the relative high MDMA loads in Amsterdam could be the presence of an extensive club scene in this STP region. This can be linked with a higher consumption of this 'party' drug. It is noteworthy that on the day before sampling, a big Halloween dance party was celebrated. Due to the travel distance of the sewer and the lag-time of the STP (24 h), sampling of the influent and effluent started when the main discharge of this party was already under treatment in the STP. In the same line, Bijlsma et al. (2009) reported high drug levels in sewage water samples due to a special music event, and suggested that these high drug levels led to a decrease in the removal efficiency.

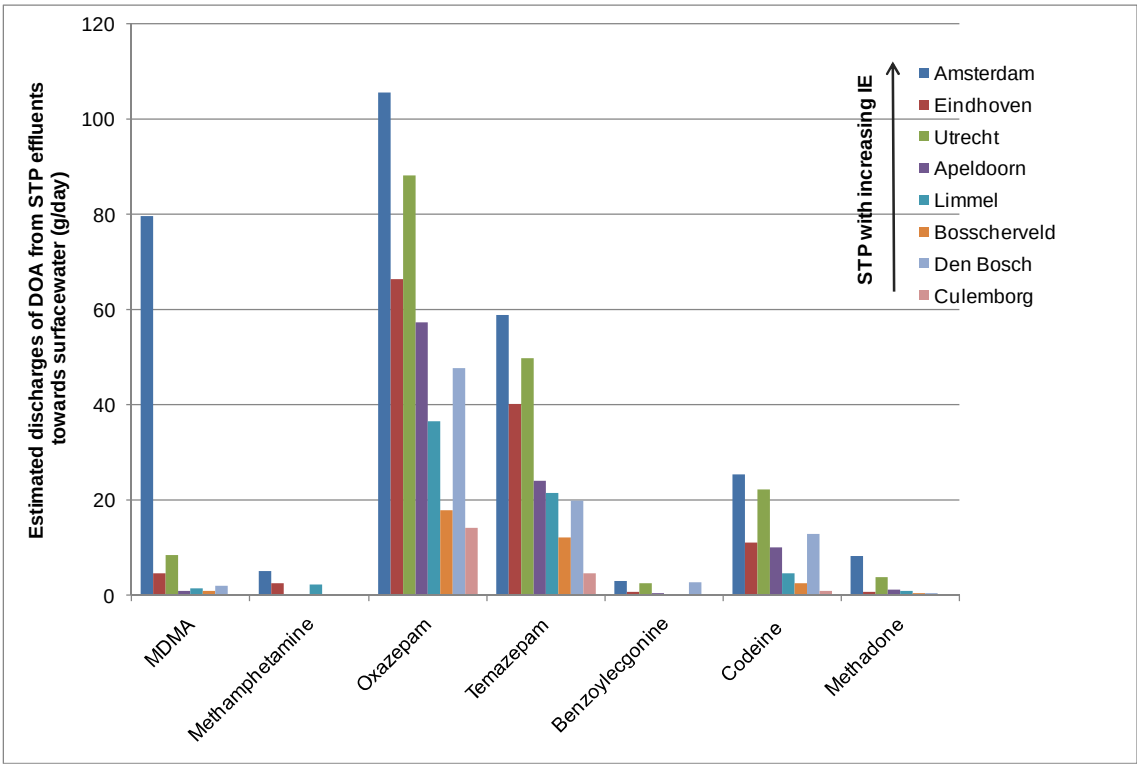

Figure 1. Estimated discharges (g/day) of DOA from STPs based on monitoring data and STP effluent flow rates in October 2009.

3.2.2. Occurrence in surface waters

In the surface waters of the rivers Rhine and Meuse, 9 DOA were detected (**Table 2**). Oxazepam, temazepam and benzoylecgonine were most abundantly present (in >70% of the sampling locations) and concentrations were highest for the benzodiazepines, with a maximum value of 68 ng/L for oxazepam. These findings are consistent with relatively high levels of benzodiazepines observed in influents and the relatively poor removal rate in Dutch STPs (Bijlsma et al., 2012). Oxazepam and temazepam were among the top 10 most prescribed pharmaceuticals in the Netherlands in 2008 (SFK, 2008). Other widely used pharmaceuticals, such as various antibiotics, beta-blockers, lipid regulators or anti-inflammatory pharmaceuticals were reported in comparable concentrations in the river Rhine (Ter Laak et al., 2010). In general, the levels of DOA and metabolites found in the river Meuse were higher than those of the river Rhine, as

shown in **Fig. 2**, most probably as a result of the larger dilution in the river Rhine which has a much larger flow rate than the river Meuse. Based on our data, loads of DOA and metabolites through the Rhine and Meuse rivers can be estimated. However it is worth mentioning that such estimations should be interpreted as indicative since they are based on grab samples and on a single sampling date.

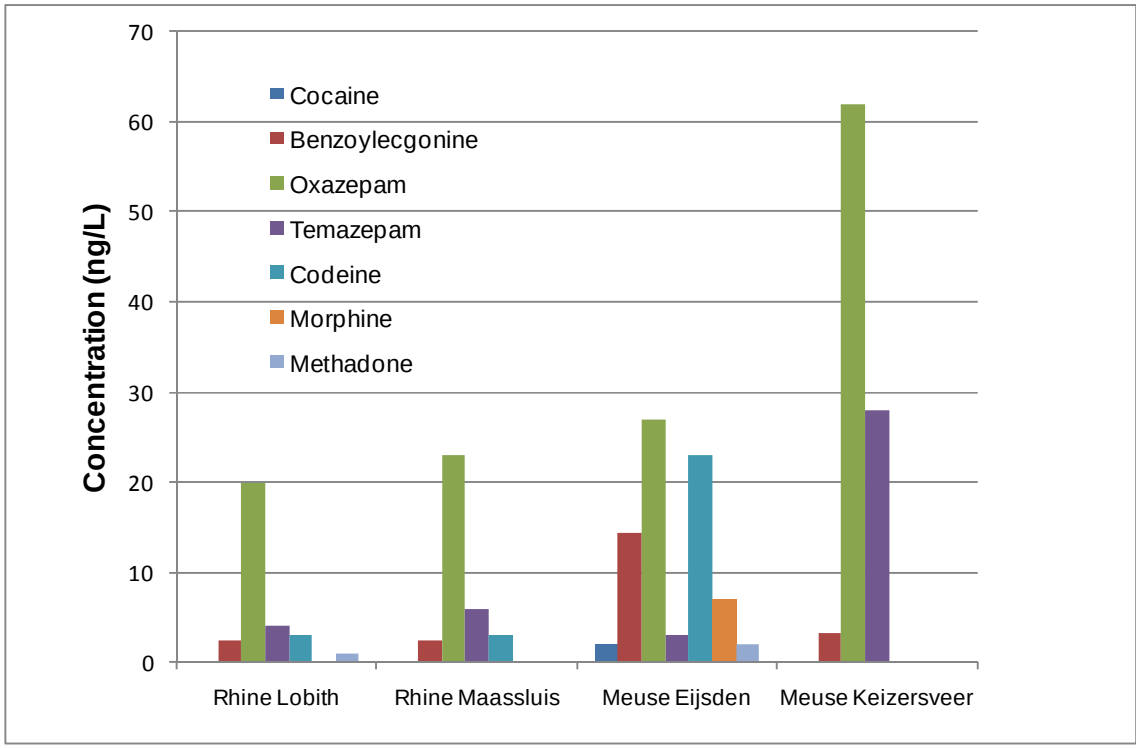

Figure 2. Concentration (ng/L) of DOA at border crossing locations (river Rhine: Lobith and river Meuse: Eijsden) and downstream (river Rhine at Maassluis and river Meuse at Keizersveer)

The loads of DOA and metabolites transported by rivers are calculated by multiplying the concentrations (ng/L) with the flow rate (L/day) recorded at the sample location on the sampling date. Flow rates on the sampling dates were obtained from the Dutch Ministry of Waterworks database. Higher flow rates in the river Rhine led to higher estimated loads in this stream (**Fig. 3** and **Table S4**). Loads were also calculated at two locations downstream: Keizersveer (river Meuse) and Maassluis (river Rhine). As shown in

Fig. 3 and **Tables S4 and S5**, the loads generally increased downstream for the four compounds presented. An increase of the riverine loads during passage of the rivers Rhine and Meuse through the Netherlands is plausible, because oxazepam, temazepam and codeine are consumed in the Netherlands in quantities of approximately 200–1500 kg per year, according to sales data from the Foundation for Pharmaceutical Statistics in the Netherlands (SFK, 2008). **Table S4** shows that for the river Rhine, the increase in loads downstream along the Dutch part of the river is of the same order of magnitude as the contribution from abroad for temazepam and oxazepam, whereas for benzoylecgonine and codeine the contribution from abroad is larger. For the river Meuse, the increase in loads for temazepam and oxazepam downstream along the Dutch part of the river seems higher than the contribution from abroad (**Table S5**). However, for the river Meuse there may also be a contribution from Belgian and German tributaries that discharge their waters into the river Meuse downstream from Eijsden. For benzoylecgonine and codeine loads are even decreasing downstream along the Dutch part of the river, which cannot be explained. Although these calculations are only indicative with considerable uncertainties, they imply that, when mitigation measures like for example improved sewage treatment are considered, these should be implemented both in Dutch and in Belgian/German STPs in order to effectively lower concentrations of DOA in Rhine and Meuse rivers. However, more data is needed to draw definite conclusions on this matter.

An attempt was made to compare the increase in loads downstream along the Dutch part of the rivers Rhine and Meuse with the loads from Dutch inhabitants in the Rhine and Meuse catchment. Bijlsma et al. (2012) showed that considerable levels of these compounds can reach the Dutch surface waters through STP effluent discharges since they are not efficiently removed in STPs. This potential contribution from Dutch inhabitants was estimated based on the average DOA loads from the 8 STPs per I.E. discharged to surface water, multiplied with the total amount of Dutch inhabitants in Rhine (ICBR, 2009) and Meuse (IMC, 2008) catchments, respectively. The calculated loads are shown in **Table S4** and **Table S5**. The increase in loads at the downstream stations Keizersveer (Meuse) and Maassluis (Rhine) should be comparable to the estimated loads from the Dutch STPs if degradation in the environment would not

occur. **Table S4** and **Table S5** however show that the loads from STPs are about an order of magnitude larger than the increase in loads at the downstream stations. This means that, despite the high insecurity of the calculations which is shown by the high standard deviations, also degradation in the environment might play a role.

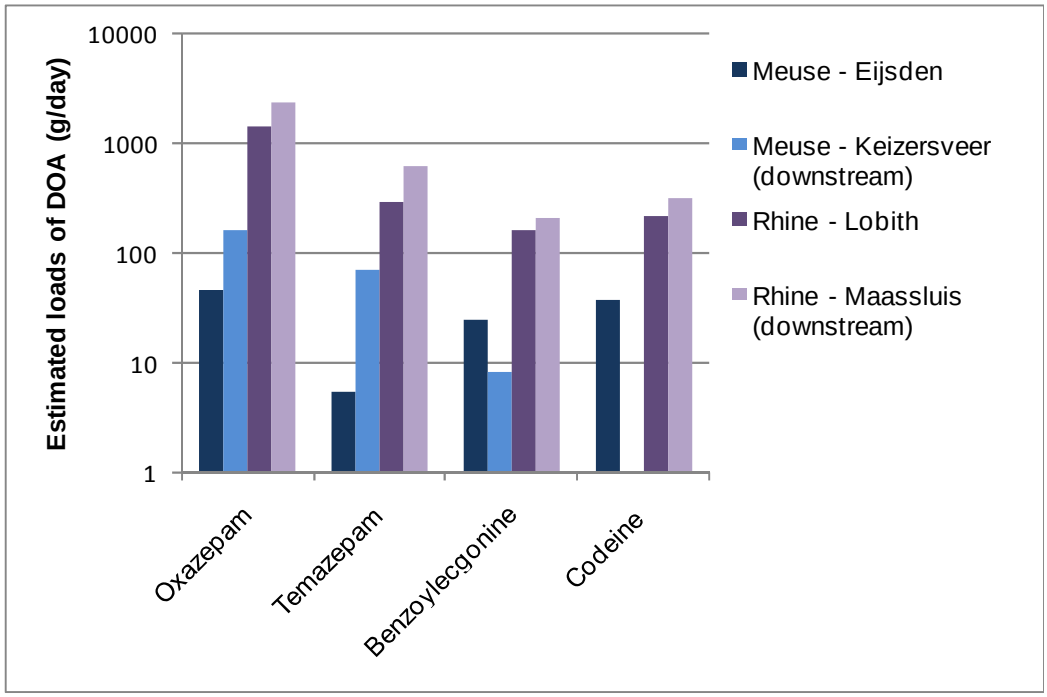

Figure 3. Estimated loads (g/day) of DOA in rivers Rhine and Meuse at Dutch border crossing locations (Lobith and Eijsden, respectively) and downstream (Maassluis and Keizersveer, respectively) calculated from monitoring data and river flow rates on one sampling date in October 2009.

3.2.3. Occurrence in the drinking water production chain

Fig. 4 presents average concentrations of DOA and metabolites observed during several stages of the drinking water production chain. Samples (from water intake locations, raw water and finished drinking water) were collected from three types of production processes where drinking water is prepared from surface waters (direct treatment and with soil aquifer recharge), and from bank filtrate. It has to be stressed here that the monitoring results are not entirely suitable to evaluate the effectiveness of

the different treatment steps, since both the raw waters and finished drinking waters were sampled only once, on the same day and without accounting for lag-times. The results should therefore be regarded as indicative, and are used here merely to provide a visualisation and qualitative assessment of compounds that are not removed completely during drinking water treatment.

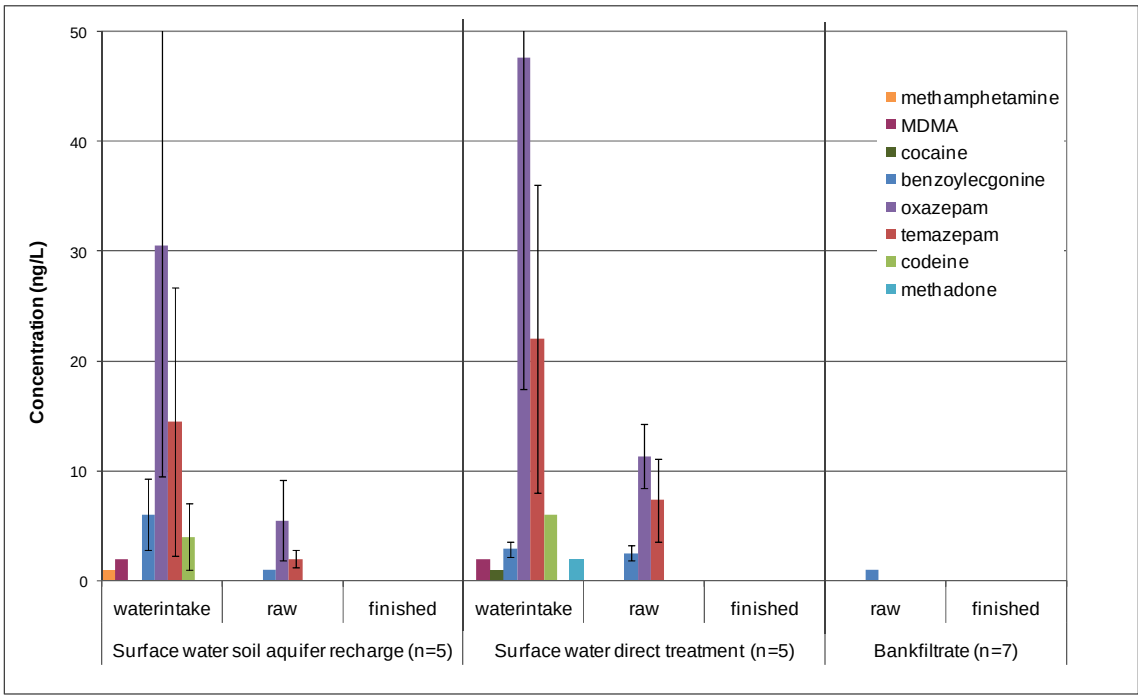

Figure 4. Average concentration (ng/L ± SD) of DOA in water collected from different stages of three types of drinking water production processes.

As shown in **Fig. 4**, amphetamine-type stimulants, cocaine and its metabolites, benzodiazepines and opiates are present in river water at the water intake locations. However, in raw water only oxazepam, temazepam and benzoylecgonine were found, and at lower concentrations. Apparently, these compounds are removed to some extent during the treatment of raw water which includes direct filtration, subsoil passage in the dune areas or storage in a reservoir. It takes place before the water enters the drinking water treatment plant where further, more advanced treatment

processes are used. Oxazepam and temazepam were not detected in the raw water that is produced from bank filtrate: possibly they were removed during bank filtration.

The treatment to produce finished drinking water mostly consists of a combination of coagulation/flocculation and filtration/flotation, UV/H_2O_2 treatment or ozonation followed by activated carbon filtration. It seemed effective in the removal of the compounds selected as none of the DOAs investigated was detected, with the exception of benzoylecgonine that was confirmed at a level between LOD and LOQ (<1 ng/L) in a single finished drinking water. Although in our study no DOA were detected in finished drinking water, Huerta-Fontela et al. (2008) did detect benzoylecgonine in Spanish drinking water. In their study on the removal efficiency of Spanish drinking water treatment plants, they concluded that benzoylecgonine was still detected in most finished drinking waters at mean concentrations of 45 ng/L, even though reductions of 90% were obtained during treatment which consists of prechlorination, flocculation and sand filtration steps. Probably the use of rather advanced drinking water treatment techniques in the Netherlands, like UV or ozonation, followed by activated carbon filtration is more effective in reducing DOA.

3.3. Environmental risk chracterization

The environmental risk characterization ratios were calculated by dividing the maximum concentrations measured in surface water (MEC) by the reported PNEC or $PNEC_{ECOSAR}$.

For oxazepam a PNEC was reported, and for codeine, cocaine, morphine, MDMA and methamphetamine QSAR derived $PNEC_{ECOSAR}$ were available (Grung et al., 2007). For temazepam and benzoylecgonine, no PNECs could be found in public literature. For temazepam however, conforming to what was done for diazepam by Grung et al. (2007), the PNEC for oxazepam was used as the default PNEC, as temazepam is also a benzodiazepine, having a similar metabolic pathway as diazepam. For benzoylecgonine, the PNEC for cocaine was used. Animal studies showed that benzoylecgonine is less toxic than cocaine, so the PNEC for cocaine will be safe for benzoylecgonine as well. For methadone no PNECs could be found or derived.

Table 3 shows the calculated MEC/PNEC ratios, which are well below 1 (range: 0.0002–0.38), meaning that, as far as data are available, no environmental effects are expected of the measured individual DOA in the surface water. However, most PNECs are derived by ECOSAR modelling and it is questionable if this is the most appropriate model. ECOSAR modelling provides acute $PNEC_{ECOSAR}$ data but with a very high degree of uncertainty. The question is whether traditional approaches to extrapolating chronic PNECs are at all relevant when considering narcotic substances. The acute/chronic ratio approach which was applied is founded on the toxic mechanism of non-specific narcosis, which is by definition not applicable to narcotics, which have a very specific effect. A high degree of uncertainty is therefore associated with the modelled acute PNEC and any assumptions made in terms of extrapolating chronic PNEC data (Grung et al., 2007). Unfortunately, no published aquatic ecotoxicological data for narcotic substances are available.

Table 3: Environmental risk characterization ratios for eight[a] drugs of abuse.

Substance	PNEC (µg/L)	Max. conc. (µg/L) in surface water (MEC)	Environmental Risk Assessment ratio (MEC/PNEC)
Methamphetamine	2.30[b]	0.001	0.0004
MDMA	2.70[b]	0.002	0.0007
Oxazepam	4.30	0.068	Σ 0.0234[d]
Temazepam	4.30[c]	0.032	
Cocaine	4.90[b]	0.003	0.0006
Benzoylecgonine	4.90[c]	0.016	0.0033
Morphine	32.0[b]	0.007	0.0002
Codeine	0.06[b]	0.023	0.3800
		Σ 0.1[d]	

[a] For methadone, which was alos detected in surface water (Table 2), no PNEC could be found.
[b] $PNEC_{ECOSAR}$, ECOlogical Structure Activity Relationships (ECOSAR) models are used to estimate PNEC.
[c] Default PNEC, set at the same level as a related compound with similar metabolic pathway.
[d] Sum of oxazepam and temazepam

3.3. Toxicological relevance for human health through drinking water

In one finished drinking water sample benzoylecgonine was detected, but at a concentration below the LOQ for this analyte (1 ng/L). Detection of this cocaine

metabolite has also been reported in Spanish drinking water although at higher concentrations, with a mean value of 45 ng/L (Huerta-Fontela et al., 2008). No other DOA were found to be present in finished water, therefore no human health risks are expected.

Currently, for individual DOA no statutory drinking water guideline values are available from e.g. European Commission, US Environmental Protection Agency (EPA) or World Health Organization (WHO). According to the Dutch Drinking Water Act a general signal value of 1 µg/L applies to organic compounds of anthropogenic origin for which no individual statutory drinking water guidelines are specified. For the twelve DOA that were detected in surface water and the five DOA that were detected in raw (process) water, the concentrations were well below this signal value. Although more research and data are needed, the results from this study suggest that the presence of DOA in drinking water should not be a cause of significant concern for human health.

4. Conclusions

This extensive screening campaign confirms the presence of DOA and metabolites at low concentration levels in the Dutch water cycle. All samples were analysed by at least three laboratories using different methodologies, a relevant and unique aspect in this type of work. DOA and metabolites were detected and quantified in sewage water influents (17) and effluents (22), surface water (9), and raw water (3). In finished drinking water only benzoylecgonine was detected in one sample, but at a concentration below the LOQ for this analyte (1 ng/L). No other DOA were found to be present in finished drinking water; therefore no human health risks are expected. Concentrations of DOA observed in surface water and raw water are well below the general signal value of 1 µg/L, which is specified for organic compounds of anthropogenic origin in the Dutch Drinking Water Act. For DOA for which an evaluation could be made, no environmental effects are expected of the measured concentrations in surface water. However further research with respect to possible long-term (chronic) effects on organisms and possible effects of combined exposure to multiple compounds at low concentrations are recommended, and the development of analytical techniques to detect possible new emerging DOA needs further attention.

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Appendix A. Supplementary information

In this section, useful information on the chemical and materials and the analytical procedure used by RIVM are given. Additionally, an overview of the sampling locations is given (Figure S1). Furthermore five tables are added: Table S1 provides the list of DOA investigated by the four participating laboratories, Table S2 shows an overview of the LOQs (ng/L) per compound, sample matrix and laboratory, Table S3 shows a comparison of results obtained by different laboratories, Table S4 compares the estimated loads entering the river Rhine through German STPs and through Dutch STPs, and Table S5 compares the estimated loads entering the river Meuse through Belgian STPs and through Dutch STPs.

Supplementary data to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.watres.2013.01.013 and in this chapter after section "References".

References

Baker, D.R., Kasprzyk-Hordern, B., 2011, Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionization tandem mass spectrometry. *J. Chromatogr. A* 1218, 1620 - 1631.

Banta-Green, C., Field, J., Chiaia, A.C., Sudakin, D.L., Power, L., De Montigny, L., 2009, The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxy-methamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater. *Addiction* 104, 1874 – 1880.

Bartelt-Hunt, S.L., Snow, D.D., Damon, T., Shockley, J., Hoagland, K., 2009, The occurrence of illicit and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska. *Environ. Pollut*. 157, 786 - 791.

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

Bijlsma, L., Emke, E., Hernández, F., de Voogt, P., 2012, Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. *Chemosphere*, 89, 1399 - 1406.

Boleda, M.R., Galceran, M.T., Ventura, F., 2009, Monitoring of opiates, cannabinoids and their metabolites in wastewater, surface water and finished water in Catalonia, Spain. *Water Res.* 43, 1126 – 1136.

Bones, J., Thomas, K.V., Paul, B., 2007, Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals. *J. Environ. Monit.* 9, 701 - 707.

Commission Decision 2002/657/EC of 12 August implementing Council Directive 96/23/EC
concerning performance of analytical methods and the interpretation of results. *Off. J. Eur. Commun.*, 2002, L221/8.

Daughton, C.G., 2001. In: Daughton, C.G., Jones-Lepp, T.L. (Eds.). Pharmaceuticals and personal care products in the environment, scientific and regulatory issues, American Chemical Society, Washington, pp. 348 - 364.

De Voogt, P., Emke, E., Helmus, R., Panteliadis, P., Van Leerdam, J.A., 2011,Determination of illicit drugs in the watercycle by LC-orbitrap MS. In Castiglioni S, Zuccato E, (Eds.) Mass Spectrometric Analysis of Illicit Drugs in the Environment. Wiley, New York, pp. 85 - 114.

Grung, M., Heimstad, E.S., Moe, M., Schlabach, M., Svenson, M., Thomas, K., Woldegiorgis, A., 2007, Human And Veterinary Pharmaceuticals, Narcotics, And Personal Care Products In The Environment. Statens forurensningstilsyn, Report 2325/2007 http://www.klif.no/publikasjoner/2325/ta2325.pdf

Hernández, F., Bijlsma, L., Sancho, J.V., Díaz, R., Ibáñez, M., 2011, Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry. *Anal. Chim. Acta* 684, 96 - 106.

Hernández, F., Sancho, J.V., Pozo, O.J., 2005, Critical review of the application of liquid chromatography/ mass spectrometry to the determination of pesticide residues in biological samples. *Anal. Bioanal. Chem.* 382, 934 - 946.

Hogenboom, A.C., Van Leerdam, J.A., de Voogt, P., 2009, Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-LTQ FT Orbitrap mass spectrometry. *J. Chromatogr. A* 1216, 510 – 519.

Huerta-Fontela, M., Galceran, M.T., Ventura, F., 2008, Stimulatory drugs of abuse in surface waters and their removal in a conventional drinking water treatment plant. *Environ. Sci. Technol*. 42, 6809 – 6816.

ICBR, 2009, Internationally Coordinated Management Plan for the International River Basin District of the Rhine. ICBR report, the Netherlands

IMC, 2008, Meuse part A. Ontwerp van het overkoepelend deel van het beheersplan voor het internationale stroomgebiedsdistrict van de Maas. Luik, 22 december 2008. Internationale Maas Commissie (in Dutch).

Irvine, R.J., Kostakis, C., Felgate, P.D., Jaehne, E.J., Chen, C., White, J.M., 2011, Population drug use in Australia: A wastewater analysis, *Forensic Sci. Int*. 210, 69 - 73.

Jones-Lepp, T.L., Alvarez, D.A., Petty, J.D., Huckins, J.N., 2004, Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents, *Arch. Environ. Contam. Toxicol.* 47, 427 - 439.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., 2009. The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during waste water treatment and its impact on the quality of receiving waters *Water Res.* 43, 363 - 380

Kvanli, D.M., Marisetty, S., Anderson, T.A., Jackson, W.A., Morse, A.N., 2008, Monitoring Estrogen Compounds in Wastewater Recycling Systems. *Water Air Soil Pollut*. 188, 31 – 40.

Postigo, C., López de Alda, M., Barceló, D., 2010, Drugs of abuse and their metabolites in the Ebro river basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation. *Environ. Int*. 36, 75 – 84.

SFK, 2008, Facts and figures. Foundation for pharmaceutical statistics. The Hague, The Netherlands.

Ter Laak, T., Van der Aa, M., Houtman, C., Stoks, P., Van Wezel, A., 2010, Relating environmental concentrations of pharmaceuticals to consumption: A mass balance approach for the river Rhine. *Environ. Int.* 36, 403 – 409.

Thomas, K.V. , Bijlsma, L., Castiglioni, S., Covaci, A., Emke, E., Grabic, R., Hernandez, F., Karolak, S., Kasprzyk-Hordern, B., Lindberg, R.H., Lopez de Alda, M., Meierjohann, A., Ort, C., Pico, Y., Quintana, J.B., Reid, M., Rieckermann, J., Terzic, S., van Nuijs, A.L.N., de Voogt, P., 2012, Comparing illicit drug use in 19 European cities through sewage analysis. *Sci. Total Environ.* 432, 432 - 439.

Van Laar, M.W., Cruts, A.A.N., Verdurmen, J.E.E., Van Ooyen-Houben, M.J.J., Meijer, R.F., 2007, The Netherlands National Drug Monitor Annual Report 2006. Published by Trimbos Institute (Netherlands Institute of Mental Health and Addiction) AF0760, 1 - 219. Utrecht, The Netherlands.

van Nuijs, A.L.N., Pecceu, B., Theunis, L., Dubois, N., Charlier, C., Jorens, P.G., Bervoets, L., Blust, R., Neels, H., Covaci, A., 2009a, Spatial and temporal variations in the occurrence of cocaine and benzoylecgonine in waste- and surface water from Belgium and removal during wastewater treatment. *Water Res.* 43, 1341 – 1349.

van Nuijs, A.L.N., Tarcomnicu, I., Bervoets, L., Blust, R., Jorens, P.G., Neels, H., Covaci, A., 2009b, Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Anal. Bioanal. Chem.* 395, 819 – 828.

van Nuijs, A.L.N., Castiglioni, S., Tarcomnicu, I., Postigo, C., Lopez de Alba, M., Neels, H., Zuccato, E., Barcelo, D., Covaci, A., 2010, Illicit drug consumption estimations derived from wastewater analysis: a critical review. *Sci. Total Environ*. 409, 3564 - 3577.

Vanderford, B.J., Snyder, S.A., 2006, Analysis of pharmaceuticals in water by isotope dilution liquid chromatography/tandem mass spectrometry. *Environ. Sci. Technol.* 40, 7312 - 7320.

Zuccato, E., Castiglioni, S., Bagnati, R., Chiabrando, C., Grassi, P., Fanelli, R., 2008a, Illicit drugs, a novel group of environmental contaminants. *Water Res.* 42, 961 - 968.

Zuccato, E., Chiabrando, C., Castiglioni, S., Bagnati, R., Fanelli, R., 2008b. Estimating community drug abuse by wastewater analysis. *Environ. Health Perspect.* 116, 1027 – 1032.

Supplementary Information

Experimental

Chemicals and materials

Drugs of abuse (DOA) and metabolites studied are listed in **Table S1**. These compounds and internal standards were purchased from Sigma-Aldrich (Madrid, Spain), Cerilliant (Round Rock, TX, USA), the National Measurement Institute (Pymble, Australia), Lipomed AG (Arlesheim, Switzerland) as solutions in methanol, ethanol, acetonitrile or as salt. Standard solutions of each compound were prepared in methanol or acetonitrile. From the standard solutions, working solutions were prepared in methanol. All standard and working solutions were stored in amber glass bottles at -18°C.

Solvents were of purity higher than 98% and obtained from Scharlau (Barcelona, Spain), Mallinckrodt Baker (Deventer, the Netherlands), Biosolve (Valkenswaard, the Netherlands) or Merck (Darmstadt, Germany). The ultrapure water was obtained by purifying demineralized water in a Milli-Q system from Millipore (Bedford, MA, USA).

Glass fibre filters (1 µm, type A/E) were purchased from Pall Corporation (Port Washington, NY, USA). Polyethersulfon filters (0.45 µm) with disposable setup were acquired from Nalgene (Rochester, NY, USA).

Solid-phase extraction (SPE) cartridges, built of a hydrophilic and a lipophilic monomer (Oasis HLB; 6 mL, 200 mg) and SPE cartridges with a mixed reversed-phase/cation-exchange sorbent (Oasis MCX; 6 mL, 150 mg or 3 mL, 60 mg) were purchased from Waters (Milford, MA, USA).

Analytical procedure RIVM

The compounds were extracted from the water samples by means of SPE. 100 mL of surface water or effluent sewage water, or five-times diluted (with demineralized water) influent sewage water were spiked with a surrogate labelled standard mix. The final concentration in the sample for each surrogate labelled internal standard was ~20 ng/L. SPE was performed using Oasis HLB cartridges that were conditioned by washing

and rinsing with 6 mL of methanol and 9 mL of Milli-Q water. Samples were percolated through the cartridges by gravity, and then vacuum dried for 15 min. Analytes were eluted using 6 mL of a 0.1% formic acid solution in methanol.

The extracts were evaporated to dryness at 35 °C under a gentle stream of nitrogen and reconstructed in 400 µL of 5% methanol aqueous solution with 0.1% formic acid. The sample extract was injected (25 µL) directly into the HPLC-MS/MS system.

Results and discussion

Comparative analysis between laboratories

From the total of 34 DOA and metabolites that were analysed in this monitoring campaign, 12 compounds were analysed by two or more laboratories. In order to compare the monitoring results of all laboratories, only the data ≥ LOQ were selected. This resulted in three compounds (benzoylecgonine, amphetamine and MDMA) and 16 samples that could be compared (6 surface water, 5 influents and 5 effluents). **Table S2** shows the average concentrations with relative standard deviation (RSD) and for each laboratory the RSD from the average concentration. With the exception of one sample, KWR detected higher concentrations, compared with the calculated average concentrations (+4 to +59% higher). Whereas mostly lower concentrations with smaller differences were detected by the other laboratories; +6 to –23% (UJI), +13 to –29% (RIVM) and –2 to –28% (UA). Differences between the laboratories are highest for two STP effluent samples for benzoylecgonine: +59% (KWR) *vs* –28% (UA) and +51% (KWR) *vs* –29% (RIVM). In general the differences are considered acceptable.

Table S1: Overview of the DOA analysed by the four laboratories.

Chemical class	Compound	Application	Internal standard	Analyzed by laboratory			
				UJI	RIVM	KWR	UA
Amphetamines	Amphetamine (AMP)	Stimulant	AMP-d_6,-d_8, -d_{11}, -d_8				
	Methamphetamine (METH)	Stimulant	METH-d_5,-d_9, -d_5, -d_8				
	MDA	Metabolite MDMA	MDA-d_5,-d_5,-d_2				
	MDMA	Empathogen	MDMA-d_5,-d_5,-d_5,-d_5				
	MDEA	Empathogen	MDEA-d_5,-d_5,-d_5				
Benzodiazepins	Diazepam	Tranquilizer	Diazepam-d_5				
	Nordazepam [a]	Tranquilizer	Nordazepam-d_5				
	Oxazepam [a]	Tranquilizer	Oxazepam-d_5				
	Temazepam [a]	Tranquilizer	Nordazepam-d_5				
	Desalkylflurazepam	Tranquilizer	Nordazepam-d_5				
	Flunitrazepam	Tranquilizer	Flunitrazepam-d_7				
Cannabinoids	Δ-9-tetrahydrocannabinol (THC)	Psychoactive substance	THC-d_3				
	11-nor-9-Carboxy-THC (THC-COOH) [b]	Metabolite THC	THC-COOH-d_3, -d_3				
	11-OH-Δ-9-THC (OH-THC) [b]	Metabolite THC	OH-THC-d_3				
Cocainics	Cocaine (COC)	Stimulant	COC-d_3,-d_3,-d_3,-d_3				
	Benzoylecgonine (BE) [c]	Metabolite COC	BE-d_3,-d_3,-d_3,-d_3				
	Cocaethylene (CE) [c]	Metabolite COC	CE-d_8				
	Norbenzoylecgonine (nBE) [c]	Metabolite COC	n/a				
	Norcocaine (nCOC) [c]	Metabolite COC	COC-d_3				
	Ecgonine methyl ester (EME) [c]	Metabolite COC	EME-d_3				
Opiates	Heroin (HER)	Psychoactive opioid	BE-d_3				
	6-monoacetyl morphine (6-MAM) [d]	Metabolite HER	6-MAM-d_3,-d_3				
	Morphine (MOR)	Opioid analgesic	MOR-d_3				
	Codeine (COD)	Opioid analgesic	6-MAM-d_3				
	Methadone	Synthetic opioid	Nordazepam-d_5, Methadone-d_6				
	EDDP	Metabolite Methadone	EDDP-d_3				
	Fentanyl	Narcotic analgesic	Fentanyl-d_5, Nordazepam-d_5				
Others	Ketamine	Dissociative hallucinogenic	Ketamine-d_4, BE-d_3				
	mCPP	Psychoactive substance	BE-d_3				
	Methcathinone	Psychoactive substance	AMP-d_{11}				
	Ritalin	Psychoactive substance	BE-d_3				
	Phenylcyclidine (PCP)	Dissociative hallucinogenic	PCP-d_5				
	LSD	Hallucinogenic	LSD-d_3				

n/a: adequate internal standard was not available. [a] metabolite of diazepam. [b] metabolite of cannabis. [c] metabolite of cocaine. [d] metabolite of heroin.

Table S2: Overview of LOQs (ng/L) per compound, sample matrix, and laboratory

Chemical class	Compound	Influent sewage water				Effluent sewage water				Surface and drinking water		
		RIVM	KWR	UJI	UA	RIVM	KWR	UJI	UA	RIVM	KWR	UJI
Amphetamines	Amphetamine (AMP)	116	42	87	2	22	2	46	2	5	1	10
	Methamphetamine (METH)	23	19	152	1	9	1	19	1	3	1	15
	MDA	324	63	160		22	2	56		5	2	17
	MDMA	41	48	76	1	11	3	9	1	2	2	10
	MDEA	46	63	154		3	2	37		2	1	13
Benzodiazepins	Diazepam		2				1				1	
	Nordazepam		4				2				1	
	Oxazepam		2				1				1	
	Temazepam		4				2				1	
	Desalkylflurazepam		1				n/b				1	
	Flunitrazepam	106				18				4		
Cannabinoids	Δ-9-THC (THC)		2375				n/b				n/b	
	11-nor-9-Carboxy-THC (THC-COOH)		152	316			28	193			10	105
	11-OH-Δ-9-THC (OH-THC)		131				13				22	
Cocainics	Cocaine (COC)	57	3	9	1	7	2	3	1	4	1	3
	Benzoylecgonine (BE)	323	5	12	1	14	2	6	1	2	1	2
	Cocaethylene (CE)			6				3				1
	Norbenzoylecgonine (nBE)			10				3				6
	Norcocaine (nCOC)			31				3				7
	Ecgonine methyl ester (EME)				2				2			
Opiates	Heroin (HER)		5				3				1	
	6-monoacetyl morphine (6-MAM)		2		1		1		1		1	
	Morphine (MOR)		4				2				1	
	Codeine (COD)		2				1				1	
	Methadone		2		1		1		1		1	
	EDDP				1				1			
	Fentanyl	417	4			4	2			3	n/b	
Others	Ketamine	51	4			8	2			2	1	
	mCPP		5				2				1	
	Methcathinone		42				2				1	
	Ritalin		5				2				1	
	Phenylcyclidine (PCP)	141				6				1		
	LSD	135				14				10		

n/b: unable to determine.

Table S3: Relative standard deviation per laboratory (%) from the average concentration of all laboratories

Water type[a]	Compound	Avg. Conc. (ng/L)	RSD[b] (%)	RSD[b] from the avg. conc. (%)			
				KWR	UJI	RIVM	UA
IWW	Benzoylecgonine	1733	16	29	-22	-4	-3
IWW	Benzoylecgonine	570	8	-3	-7	13	-3
IWW	Benzoylecgonine	1193	17	23	-1	-5	-17
IWW	Benzoylecgonine	2907	10	12	6	-5	-13
EWW	Benzoylecgonine	21	38	59	-23	-8	-28
EWW	Benzoylecgonine	26	38	51	-2	-29	-21
EWW	MDMA	54	26	33	-20	9	-22
EWW	MDMA	92	24	35	-9	-4	-22
EWW	MDMA	537	20	28	-8	-1	-20
IWW	Amphetamine	107	8	4	-11	9	-2
surface water	Benzoylecgonine	14	15	5	-16	12	-
surface water	Benzoylecgonine	2,3	26	29	-14	-14	-
surface water	Benzoylecgonine	2,3	26	29	-14	-14	-
surface water	Benzoylecgonine	2,3	26	29	-14	-14	-
surface water	Benzoylecgonine	3,3	18	20	-10	-10	-
surface water	Benzoylecgonine	8,3	7	8	-4	-4	-

[a] IWW: influent wastewater; EWW: effluent wastewater.
[b] RSD: relative standard deviation

Table S4: Estimated loads of DOA (g/day) that enter the Netherlands from Germany through the river Rhine and downstream, and comparison with loads from Dutch (NL) inhabitants in Rhine catchments through STP effluents.

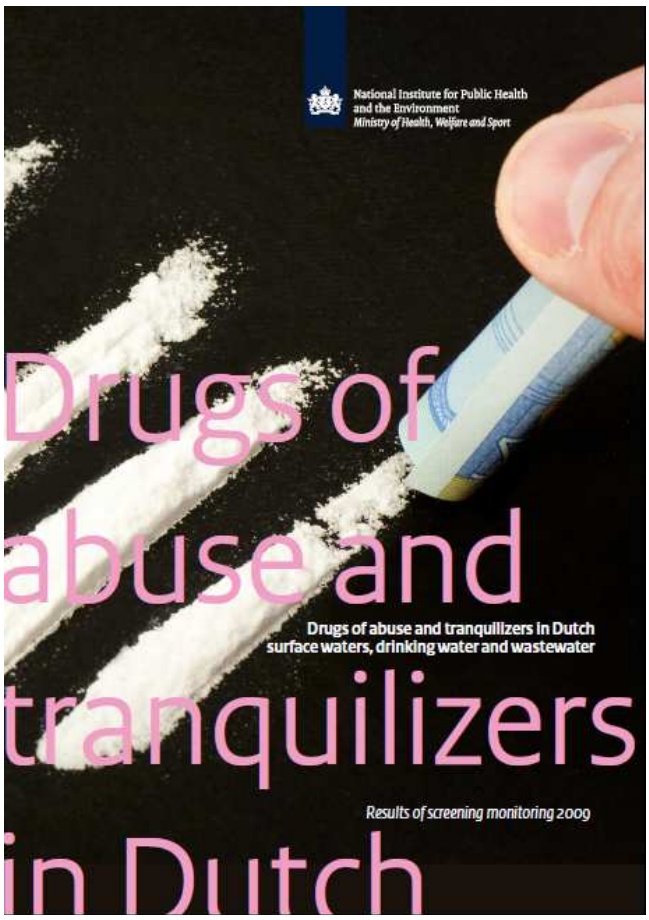
Compound	Rhine – at border (Lobith)	Rhine- downstream (Maassluis)	STP effluent load NL Rhine	St.dev
Oxazepam	1460	2427 (+967)	7745	11579
Temazepam	293	633 (+340)	3104	3852
Benzoylecgonine	170	211 (+41)	193	347
Codeine	219	318 (+99)	1109	1526

Table S5: Estimated loads of DOA (g/day) that enter the Netherlands from Belgium through the river Meuse and downstream, and comparison with loads from Dutch (NL) inhabitants in Meuse catchments through STP effluents.

Compound	Meuse – at border (Eijsden)	Meuse – downstream (Keizersveer)	STP effluent load NL Meuse	St.dev
Oxazepam	47	162 (+115)	2222	3322
Temazepam	6	71 (+65)	891	1105
Benzoylecgonine	25	8 (-17)	55	100
Codeine	38	0 (-38)	318	438

Figure S1: Overview of sampling locations of the monitoring campaign on DOA in Dutch waters. Colored regions correspond to water suppliers (van der Aa et al., 2010).

Scientific article 10 is a result of a collaborative study between the National Institute for Public Health and the Environment (RIVM), KWR Watercylce Research Institute, University of Antwerp and the University Jaume I. This work also resulted in a RIVM Report 703719064/2010 "Drugs of abuse and tranquilizers in Dutch surface waters, drinking water and wastewater" coordinated by the RIVM.



REFRENCES

Van der Aa NGFM, Dijkman E, Bijlsma L, Emke E, van de Ven BM, van Nuijs ALN, de Voogt P. 2010, Drugs of abuse and tranquilizers in Dutch surface waters, drinking water and wastewater. RIVM report 703719064, Bilthoven, 1-90. Available from URL: http://www.rivm.nl/dsresource?objectid=rivmp:26501&type=org&disposition=inline&ns_nc=1

5.3 Discussion of the results

Occurrence of illicit drugs in the aquatic environment

Several studies dealing with the analysis of IDs in the aquatic environment have been reported in the last few years. However, for 5 to 10 years ago, little was known about the occurrence of IDs and their metabolites in the water environment. In Spain, parallel to our studies (*scientific article 1* and *scientific article 8*), other research groups also reported the occurrence of IDs in wastewaters, surface waters [Huerta-Fontela *et al*. 2007; Boleda *et al*. 2009; Gonzalez-Mariño *et al*. 2010; Postigo *et al*. 2010; Vazquez-Roig *et al*. 2010; Pedrouza *et al*. 2011]and even drinking waters [Huerta-Fontela *et al*. 2008a]. In the Netherlands, exploratory studies on their presence in WWTP effluents, surface waters and drinking waters were conducted in the period 2006–2007 by the KWR Watercycle Research Institute (Kiwa Water Research at that time) [Hogenboom *et al*. 2009; De Voogt *et al*. 2011]. However, at that time, concentration levels could not be calculated as KWR did not possess a license to order and store IDs standards. Yet, the detection of some drugs induced the more detailed monitoring campaign in the Netherlands presented in this chapter (*scientific article 9* and *scientific article 10*).

A one-to-one comparison of data with the literature is difficult to make, and precaution on the interpretation of the data is required. For a correct comparison of data, various factors (e.g. sampling, treatment processes, stability of compounds, analytical measurement etc) and the uncertainties associated to the different steps need to be taken into account. This would implicate a more extensive study, which is a complex task that requires much more research and financial support. However, a rough comparison of data can be performed, keeping the limitations in mind. In general, the concentration levels of the IDs and metabolites investigated were approximately of the same order of magnitude as those published in other countries [Castiglioni *et al.* 2011]. The lowest concentrations, as expected, were detected in drinking- and surface waters, while the highest ones were observed in wastewater influents.

Occurrence of illicit drugs in influent wastewater samples

In influent wastewater samples from both Spain and the Netherlands, cocaine and its metabolite BE showed the highest mean concentrations, with significant higher levels observed for BE compared to cocaine. BE is therefore commonly used in SBE for more reliable and accurate calculations of cocaine use. In general, cocaine and its metabolites also belong to the group of the most studied IDs in the aquatic environment. The concentration range in our works, from 100 ng/L to 3000 ng/L, is in agreement with levels reported in other monitoring studies in Spain [Huerta-Fontela *et al*. 2007; Postigo *et al*. 2010], but also in Belgium [van Nuijs *et al*. 2009], Croatia [Terzic *et al*. 2010], France [Karolak *et al*. 2010] and Italy [Castiglioni *et al*. 2006]. Amphetamine, methamphetamine, MDMA and THC-COOH were the other drugs investigated in all three studies presented in this chapter. Amphetamine and MDMA were frequently detected in influents from the Netherlands. However in Spain (Castellón province), these designer drugs were occasionally detected in weekends, during holiday periods and/or during a pop/rock festival. These drugs were also found in 30% of the influent samples (n = 16) in Northeast of Spain [Huerta-Fontela *et al*. 2007] and in more than 90% of the influent samples (n = 14) of the Ebro river basin area [Postigo *et al*. 2010]. The latter also reported methamphetamine in two of their samples analyzed at very low concentration levels (0.8 and 8.4 ng/L). In our studies, methamphetamine was quantified at 16 and 17 ng/L in two samples from Schiphol international airport, and at 24 and 278 ng/L in two influent samples from Eindhoven and Maastricht (Limmel), respectively. A plausible explanation for the generally low detection frequency and low concentrations found, might be that consumption of this drug in Europe less popular is [UNODC 2013]. For example, in the USA methamphetamine was frequently detected in sewage water and at higher concentration levels, up to 550 ng/L [Chiaia *et al*. 2008]. The drug biomarker for cannabis, THC-COOH, was found in most of the influent wastewaters samples of the Dutch cities analyzed at a concentration range of 91 - 375 ng/L. In Spain, concentration levels were generally much lower < 40 ng/L [Boleda *et al*. 2007; Postigo *et al*. 2010], and in Castellón, THC-COOH could only be quantified in the influent samples taken during the music event. Analytical limitations of the methodology surely played an important role in the low frequency of detection of this compound. Recent improvements in the analytical instrumentation (*i.e*. newly designed

Step-wave ion guide incorporated in the LC-MS/MS instrument) have facilitated the determination of THC-COOH in water samples from Castellón in future samples [Bijlsma *et al*. 2014 – *scientific article* 2].

An interesting aspect within our studies was the results obtained from samples taken during special events. Higher concentrations of the party drug MDMA (ecstasy) were linked to a Halloween dance party in Amsterdam and considerable increase and a clear short-time change in drug use were observed in Spain (Benicassim) during a big international pop/rock festival (FIB). Cocaine, BE, amphetamine, MDA and THC-COOH were found at high concentrations during this period. However, the most relevant fact was the extraordinary increase of MDMA, which was only very little detected during the weekends (data shown in ***figure 1, scientific article 8***). The FIB in 2008 was mainly visited by young Spanish and Britisch music fans. The United Kingdom is reported to have a high prevalence of ecstasy use among young adults (aged 15 - 34) [EMCDDA, 2012], which is also reflected with high per capita loads detected in London [Thomas *et al.* 2012]. Hence, the high MDMA concentrations found during the festival might be related to the mostly British visitors. Interesting data were also obtained from Schiphol international airport and Eindhoven, where direct disposal of cocaine and amphetamine was suggested, respectively. The latter was also linked to a possible elevated consumption during the carnival period. In order to determine if amphetamine proceed from consumption or an incidental dump, enantiomeric profiling may be very useful. This was also suggested for data obtained from Benicassim, where the presence of MDA could be due to consumption of MDA itself, or from the consumption or transformation in the sewage system of MDMA. Distinguishing between the enantiomeric forms of chiral drugs has proven to be very helpful in these cases [Kasprzyk-Hordern and Baker 2012].

<u>Occurrence of illicit drugs in effluent wastewater samples</u>

The analysis of effluent wastewaters (*i.e.* samples taken from the outlet of a WWTP) provides useful information on removal of IDs during wastewater treatment processes and on the possible discharge of these compounds into the aquatic ecosystem. The **removal efficiency** (RE) of a WWTP is reflected by its capacity to eliminate organic

contaminants and has been estimated from IDs concentrations found in influents (Ci) from day (x) and from their corresponding effluents (Ce) from day (x+1), assuming an average residence time of water in the plant of 24h. Removal efficiencies are than estimated using the following equation:

$$RE = (1 - (Ce/Ci)) \times 100\%$$

Applying this approach, only a rough estimation is obtained, as the potential "losses" of drugs associated to the solid particles are not taking into account. This variable was not included in this work, as suspended particular matter was not analyzed for IDs. Removal efficiencies varied from 100% elimination, when analytes were detected in influent waters but were absent in effluents, to 0% elimination, when analytes were present in influents and in effluents at around the same level or when analytes were not detected in influents. "Negative" elimination rates were considered when analyte concentrations were found higher in the effluent.

The removal by WWTPs depends on several factors such as type of wastewater treatment technology implemented, residence time of the water in the WWTP, weather conditions at time of sampling, etc. Thus, several uncertainties are associated to the calculations and therefore more data is needed to allow better and more accurate estimates. Nevertheless the rough estimates presented in the first two studies gave a useful indication on the performance of the WWTPs in Spain and the Netherlands. It is worth noting that efficiencies could not be estimated for the WWTPs studied in *scientific article 10*, due to the fact that samples were taken on the same day, without accounting for the residence time.

All WWTPs investigated were equipped with conventional activated sludge secondary treatment. The main differences between them refer to their water treatment capacity, residence time and additional treatment processes. The different meteorological conditions between the Netherlands and Spain and between winter and summer did not seem to be an important factor that affects bioactivity/degradation. In general, removal of the IDs by the WWTPs from Spain and the Netherlands compares well and were in agreement with other reports [Huerta-Fontela *et al*. 2008a; Postigo *et al*. 2010;

Terzic *et al*. 2010; Baker and Kasprzyk-Hordern 2013]. Removals of amphtemine, cocaine and its metabolites and THC-COOH were usually higher than 75%, and MDMA presented in both studies high variability with normally poor elimination (< 40%). (Data of removal efficiencies of IDs and their metabolites by Spanish and Dutch WWTPs can be found in ***figure 3 of scientific article 8*** and ***figure 2 of scientific article 9***, respectively). Opiates, studied in the Netherlands, also showed high differences and lower removal efficiencies, and benzodiazepines were commonly found at higher concentrations in effluents than in influents. The removal of IDs by WWTPs during high peaks of drug usage was not previously evaluated in the literature, but the increased consumption during the FIB festival showed to have a negative effect on the efficiency by the WWTP of Benicassim.

The daily concentration of each drug detected in effluent wastewaters also permited an estimation of their discharges towards receiving natural waters. **Loads of illicit drugs** discharged via sewage effluents to the Mediterranean Sea and to the rivers Rhine and Meuse were calculated by multiplying the concentrations (ng/L) found with the daily flow (m^3) of effluent water reported by the WWTPs (***table 1 of scientific article 8*** and ***figure 1 of scientific article 10***). In general, higher loads were calculated in Amsterdam and Castellon, *i.e.* in more urbanized areas with higher inhabitant equivalents under study and where higher consumption is expected. Both studies also showed a noticeable increase in the discharge of IDs during two special music events. Hence, if removal efficiencies (%) are more or less constant, higher discharges can be expected when average drug consumption per inhabitant increases.

<u>Occurrence of illicit drugs in surface water and drinking water samples</u>

Incomplete removal during wastewater treatment is directly related to the presence of IDs and metabolites in surface waters. Surface waters and raw and finished drinking waters from the Netherlands were screened for IDs in *scientific article 10*. In surface waters of the rivers Rhine and Meuse, cocaine and BE were detected at levels up to 16 ng/L, whereas the pharmaceutical with potential abuse *i.e.* codeine, oxazepam and temazepam were detected at levels up to 23, 68 and 32 ng/L, respectively (***table 2 of scientific article 10***). Cocaine and BE, the most abundant ID in wastewater, were also

found in the Spanish Ebro River basin and reached 59 and 346 ng/L, respectively [Postigo *et al.* 2010]. In Italian surface waters these compounds were also reported up to 44 and 183 ng/L, respectively (Zuccato *et al*. 2008a]. It is difficult to perform realistic comparative studies. For example, information on the exact discharge and sampling points would be necessary as levels of IDs were reported to significantly decrease with increasing distance from the discharge point [Bones *et al*. 2007]. Rivers in their lower course present usually higher dilution factors than upstream. However, levels of ID loads in Dutch rivers generally increased from upstream to downstream, similar which was observed in the Spanish Llobregat river basin [Huerta-Fontela *et al*. 2008b; Boleda *et al*. 2009]. The contribution from inhabitants via sewage effluents downstream might play an important role.

Although it is complicated to compare data, the results obtained gave information on the occurrence and fate of IDs in the Dutch surface waters and contribute to a better understanding of its environmental importance. Furthermore, drinking water was also analyzed as approximately 40% of the drinking water of the Netherlands is produced from surface water. Only one positive sample was found in this case, corresponding to one finished drinking water sample where BE was detected, although at a concentration below the LOQ (1 ng/L). No other drugs were found to be present in finished drinking water.

Based on the measured concentrations in surface water and drinking water in this work, neither environmental effects nor human health risks are expected, at this point. Nevertheless, alertness is required and ongoing research with respect to possible effects of combined exposure to multiple compounds at low concentrations needs attention. The development of highly sensitive analytical methods is also required to detect possible new emerging contaminants in drinking water. In general, more thorough monitoring campaigns are needed.

Uncertainties associated

The results obtained from the analysis of sewage water give useful information on trends of drugs usage from the population as demonstrated previously. In order to constrain

over- or under-reporting of drug concentrations and produce more reliable and comparable estimates of ID use, uncertainties associated to the determination of IDs in sewage need to be reduced [van Nuijs *et al*. 2011a; Castiglioni *et al*. 2013]. Most limitations identified and recognized for the SBE approach also affect the quality of data in wastewater effluents and surface waters. A reliable determination of IDs in these matrices permits also a better and more realistic knowledge on removal and on the possible environmental impact.

In the introduction of this chapter we discussed several issues regarding sampling, stability of compounds and analytical measurement. In order to limit the uncertainties related to these aspects and reduce over- or under-reporting of drug concentrations, precaution was taken in our work. In some occasions new research also forced us to modify certain precautionary measures and allowed better estimates.

Sampling

24-h flow dependent samples from influent and effluent of the Netherlands were collected. The sampling uncertainty associated to these samples was expected to be less than 5% on an individual day [Castiglioni *et al*. 2013]. This was calculated with conservative assumptions on prevalence and number of relevant toilet flushes according to Ort *et al*. 2010. In Spain, however, the sampling error may exceed this value due to a relative high time proportional sampling interval (every hour). In *scientific article 8* the sampling error was estimated to be 30%. Based on this work, the uncertainty on the data reported in *scientific article c* could be reduced by taking a sample every 15 min.

Uncertainty is high when taking a single grab sample (**Table 5.1**) as occurred for the Dutch surface water samples. However, the objective in *scientific article 10* was to provide an indication on the occurrence of IDs in these waters. A new set of experiments would be necessary to evaluate, for example, daily or seasonal variations.

Compound stability

The stability of IDs in sewage is an important issue. Amphetamine, methamphetamine, MDMA and methadone are generally stable up to 12 h at 20 °C, but for 6-MAM, a

minor but exclusive metabolite of heroin, a significant loss (20%) was observed [van Nuijs *et al*. 2012b]. Castiglioni *et al*. (2006) also observed high stability for amphetamines, ATS and THC-COOH at 4 °C for several days. Less information is available for the other opiods, ketamine, methylphenidate, benzodiazepines, mCCP and fentanyl included in *scientific article* 9 and future research is necessary with regards to their stability in wastewater. Furthermore, cocaine, norcocaine and cocaethylene are not stable under the conditions (decrease > 10%) studied, and can transform into BE and norBE through chemical hydrolysis [Castiglioni *et al*. 2013]. In general, the bias due to cocaine transformation is expected to be relatively low (< 10%) as the estimated mean residence time of wastewater in the sewers investigated is < 6 h and the conditions for sampling and sample storage minimize degradation of IDs and metabolites.

As previously stated the removal efficiency has been estimated using concentrations of analytes determined in solely wastewaters. The lower levels commonly found in effluents are assumed to be a result of removal in the WWTP, due to microbial degradation, or other transformation processes. However, the analysis of suspended particular matter (SPM) has also been suggested to prevent under-reporting. The analysis of both sewage water (influent and effluent) and SPM provides a better estimation and more realistic knowledge on removal and environmental impact of compounds by WWTP systems, as removal from wastewater does not necessarily mean degradation (Baker *et al*. 2012; Baker and Kasprzyk-Hordern 2013). For most IDs included in our studies, less than 5% was estimated to be present in SPM (Baker *et al*. 2012), thus under-reporting was assumed to be relatively low. However, some drugs such as THC-COOH were not included in the above mentioned work, and for this compound partition to SPM might be greater compared to the other selected drugs, owing to its less polar character.

Analysis: quantification, confirmation and quality assurance

The focus of this PhD thesis lies on analytical chemistry, therefore special emphasis was placed on the reliable quantification and confirmation of the compounds detected. The analytical determination of IDs and metabolites in water samples requires a certain sample preparation (commonly analytes pre-concentration) owing to the low

concentrations (ng/L) range that have to be measured in a complex matrix. This thesis demonstrates that a single concentration and clean-up step based on off-line SPE and analysis with LC-MS/MS or LC-HRMS are efficient for the trace determination of these compounds in wastewater (influent and effluent) and surface water (*Chapter 2* and *Chapter 3*).

The determination of low concentrations of IDs and their metabolties in complex water samples is an analytical challenge. The replicate analysis of samples based on individual extractions, the use of analyte own ILIS, and the application of criteria based on acquisition of accurate mass data (e.g. < 5 ppm in HRMS) or several MS/MS transitions (in LR tandem MS) and on their specificity, retention times and ion intensity ratios are of particular importance for reliable quantification and confirmation. Owing to the absence of guidelines in this field, the proposed quality procedures recommended in other research fields [2002/657/EC; SANCO/12495/2011], were also applied in this work. However, these procedures might not always be realistic in sewage water sample analysis. Especially, the compliance of ion ratio deviation might be problematic. Theoretical ion ratios, obtained from standard solution in solvent, did not seem concentration dependent, but they seemed to differ depending on the instrument used and on the parameters optimized (e.g. cone and collision voltages), while ion ratios in samples might also be affected by matrix components. We therefore recommended that ion ratios are tested and accomplished within each sequence using the standards included in every sequence of sample analysis. Using this approach, the wide majority of positives were confirmed by accomplishment of Q/q ratio. Maximum tolerances permitted depended on relative ion intensity and varied between 20 to 50% [2002/657/EC]. Yet, this parameter was in some cases problematic, mainly at low analyte levels, but also at higher analyte levels as shown in **Figure 5.1**.

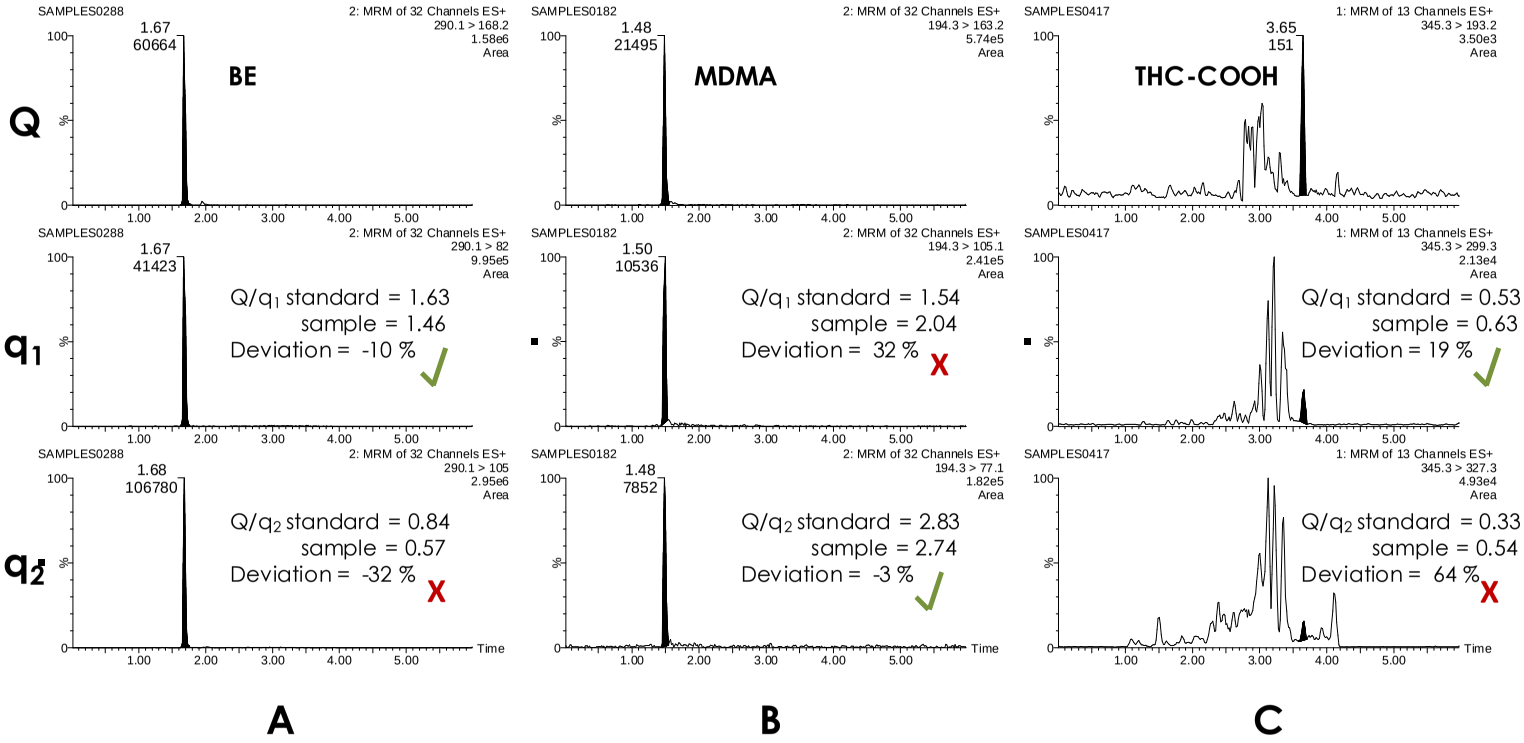


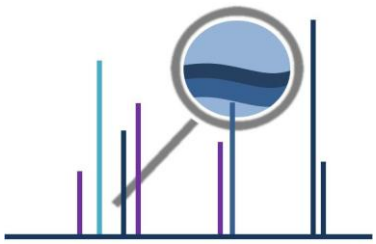
Figure 5.1: Non-compliance of one Q/q ratio, UHPLC-MS/MS chromatograms corresponding to the positive finding of A) benzoylecgonine 2354 ng/L in IWW, B) MDMA 476 ng/L in EWW, C) THC-COOH 1638 ng/L in EWW. (Q) quantification transition; (q_1) and (q_2) confirmation transitions.

The non-compliance of the ion ratio might be due to the presence of matrix components sharing one of the two transitions, thus affecting the Q/q ratio. To deal with this situation different options can be recommend regarding LC-MS/MS analysis: 1) test Q/q ratios of additional transitions acquired (this means that more than two transitions should be acquired for each compound, which was in fact made in the developed methodologies reported in chapter 2); 2) re-inject the sample and re-calculate Q/q ratios; 3) modify chromatographic conditions trying to avoid interfering peaks at any of the two transitions; 4) consider the possibility of injecting the sample extract using another analytical technique such as HR MS (accurate mass measurement), if initially analyzed with triple quadrupole mass analyzer. In the case that no additional information can be obtained, the sample might be reported as positive, but including a comment on non-compliance of the ratio, and reporting the Q/q ratio deviation obtained. In these cases, additional work would be necessary to test if any interfering compound was affecting the Q/q ratio.

Another aspect often unreported or not treated in detail in the scientific literature is the application of quality controls, both internal and external. Routinely, internal quality controls (QCs) are analyzed to test for daily method variations and method robustness. To each sample batch (max. 10 samples), one QC in sample was included in this thesis. A blank sample was difficult to obtain, therefore a sample with lowest concentrations expected was selected (e.g. Wednesday). The QC together with the non-spiked "blank" sample were analyzed in the same batch. The concentration values found in the non-spiked sample were subtracted from the QC to obtain recoveries. In this way, better insight of the data was obtained and possible errors might be explained (e.g. non-compliance of confirmation criteria might occur due to matrix interferences). In the light of realistic data obtained for QCs, acceptable ranges were considered between 60-130% to give the sample sequence analysis as satisfactory.

Finally, in this thesis external quality controls were analyzed using the developed methods described *scientific articles 1*, *2* and *5*. The external quality controls formed part of inter-laboratory comparison exercises carried out in 2012 and in 2013. The results we reported in two collaborative studies presented in chapter 6 of this thesis (Castiglioni

et al. 2013 - scientific article b; Ort *et al.* 2013 - scientific article c). Z-scores obtained by our laboratory were satisfactory (-2 < Z < 2) for the analytes included; amphetamine, methamphetamine, MDMA, cocaine, BE and THC-COOH. Z-scores are an accepted measure for the performance of an individual laboratory with regard to the group average [UNIDO 2006]. Inter-laboratory comparison studies are very useful and highly recommended for regular checks of quality control as they give relevant information on the performance of the methodology applied.



CHAPTER 6

European-wide collaboration

6.1 Sewage analysis CORe group Europe

The Sewage analysis CORe group Europe (**SCORE**) network was established in 2010 to bring together EU research groups working in the field of sewage-based epidemiology of illicit drugs (**Fig. 6.1**). The initial goal of this group was to collaborate on international studies comparing ID use between cities and time periods, and to evaluate and harmonize the different analytical procedures being used.

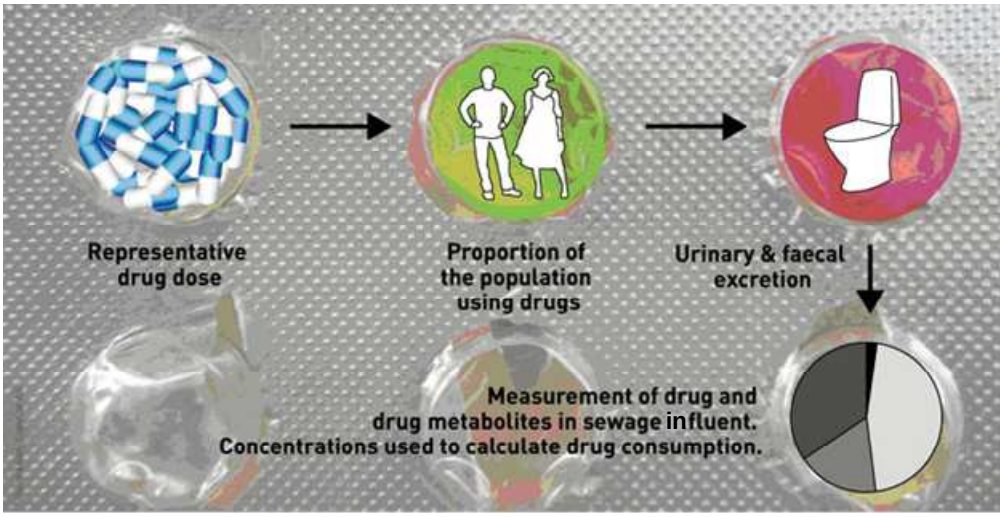


Figure 6.1: Sewage-based epidemiology [http://www.niva.no/SCORE].

Since then, the SBE approach has developed rapidly providing near-real time information on ID use in Europe. The SCORE group has coordinated three monitoring

studies on the temporal and spatial differences in illicit drug use in European cities (Thomas *et al.* 2012 - *scientific article a*; Ort *et al.* 2014 - *scientific article c*). The first study was performed in 2011 including 19 cities, and it was followed-up in 2012 and 2013 with 23 and 42 cities, respectively. The quantitative measurements provided important additional, complementary and the most actual information on ID use in Europe through the analysis of sewage water. The potential of the approach was recognized by the EMCDDA and the UNODC and established a closer collaboration and communication with experts from different research fields. The development of the program has been strategically managed with input from these end-users and policy makers to ensure that the additional countries and drugs included were of value.

The use of an agreed best-practice consensus document for sampling, storage and analysis and the performance of inter-laboratory data quality exercises coordinated by the SCORE group became essential. The discussion and evaluation of outcomes were highly valuable for each participating research group and of great importance in order to produce reliable and comparable results within each monitoring. Another major step was the involvement of sewer engineers to evaluate the influence of different sewer designs and sampling procedures on the data generated. This has not only allowed the uncertainty associated with sewers and sampling to be evaluated but has also allowed a comprehensive characterization of the major uncertainties of the approach and points for further improvement (Castiglioni *et al.* 2013 - *scientific article b*).

As result of our research, different national and international institutions are now interested in applying wastewater analysis as a new tool. It has been acknowledged that it can provide valuable additional information that is complementary to quantifying prevalence of illicit drug use by means of infrequently carried out population surveys.

Coordination efforts

The network's activities are coordinated by the SCORE group members made up of Sara Castiglioni (Mario Negri Institute), Alexander van Nuijs, Adrian Covaci (University of Antwerp), Erik Emke, Pim De Voogt (KWR), ***Lubertus Bijlsma***, Félix Hernández (University

Jaume I), Christoph Ort (Eawag), Barbara Kasprzyk-Hordern (University of Bath), Malcolm Reid and Kevin Thomas (NIVA).

Coordinating such transnational and multidisciplinary research action is not an easy task, especially without direct funding. The SCORE group has been supported by the immense good will of all its participants, supplemented by small amounts of ad hoc funding from the Norwegian Research Council and the EMCDDA.

As the responsible European Agency for reporting factual information on illicit drugs it has been important for the SCORE network to work closely with the EMCDDA to gain acceptance of the technique and start to work closely with the epidemiologists whom are responsible for estimating the scale of Europe's drug problem. Nowadays, the SCORE network includes experts working in relevant research fields such as pharmacokinetics, drug epidemiology, forensic science, analytical chemistry, and sewer engineering.

Dissemination

The SCORE network has thus far been an outstanding success with a number of notable highlights, which include three European-wide surveys involving up to 42 cities providing the most recent data on the illicit drug situation in Europe. This effort of SCORE has also resulted in three joint articles published in peer-reviewed scientific journals, as mentioned above and presented in section 6.3. This success was best exemplified by the global media coverage of our first European study during the summer of 2012 (**Fig. 6.2**). In addition, three workshops and the first international multidisciplinary conference on detecting illicit drugs in wastewater: "*Testing the waters*" were organized in collaboration with the EMCDDA. Furthermore, the SCORE group coordinated two inter-laboratory comparison exercises and drafted a best-practice advice on the collection and analysis of sewage samples. Finally, effort of the SCORE group resulted in European funding (section 6.2), which will ensure continuous research by the network and to build upon its existing achievements.

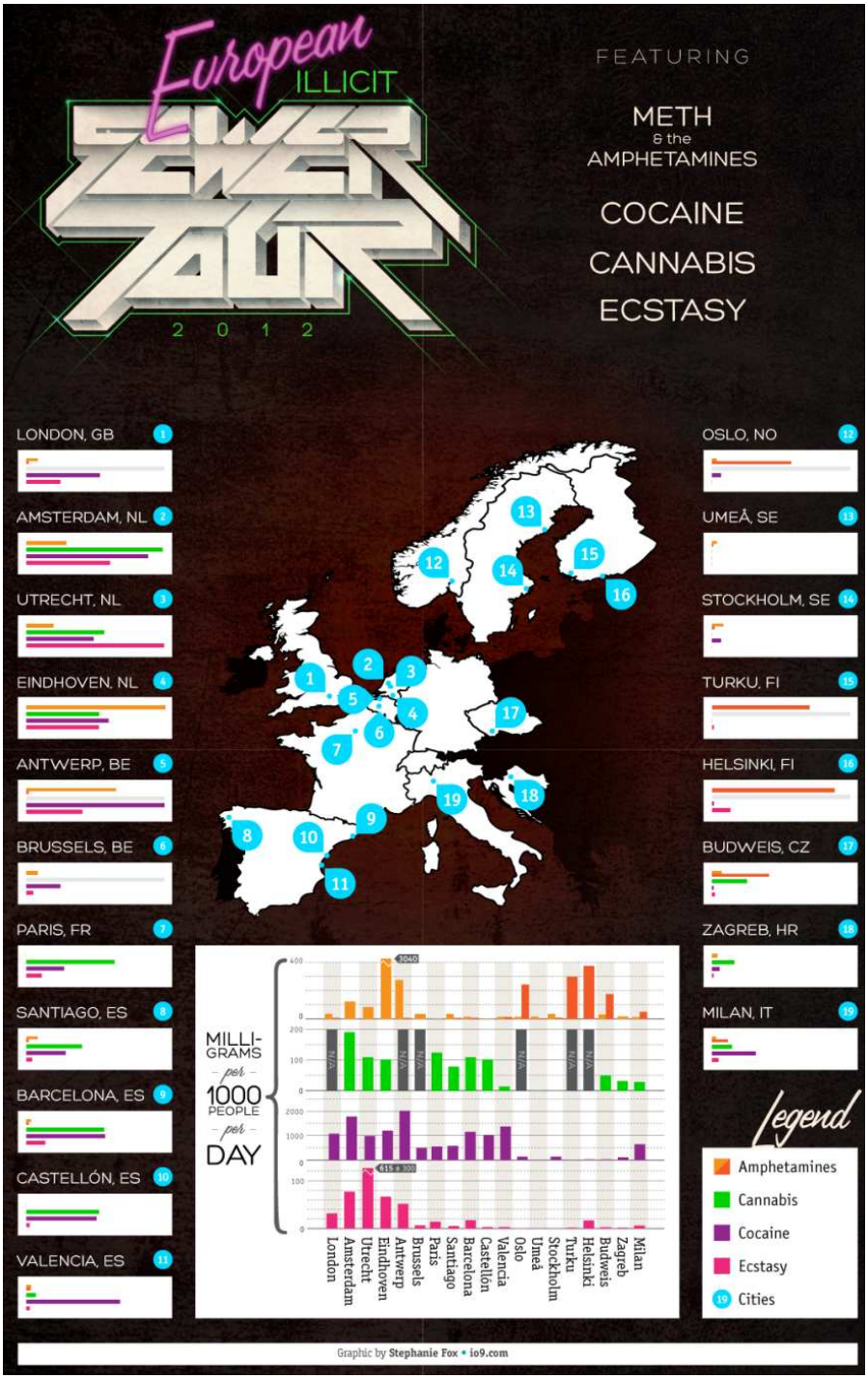


Figure 6.2: Example of global media coverage [http://www.io9.com].

Future perspectives

The SCORE group will continue to coordinate European, and potentially broader, comparative studies, along with laboratory performance studies whilst liaising with the EMCDDA and other international governmental agencies such as the UNODC. SCORE believe that measurements in the sewers are an important addition to the methods that exist today for estimating drug prevalence and that one day soon the data will be commonly used alongside questionnaire based approaches. Furthermore, SBE has the potential to be expanded to other aspects of public health such as nutrition, allergies, diseases, lifestyle, etc. The main benefit is that small numbers of samples cover large populations and few legal or ethical obstacles are encountered as no individual can be identified.

The scientific challenge of the network will focus towards the robustness and a better understanding of the uncertainties associated with the proposed methodologies. Members of SCORE are among the main drivers of SBE globally. Their goal is to unite leading experts from different fields and sectors (incl. academia, governmental agencies and private sector) to enhance the trans-disciplinary thinking. This will facilitate transferring research into practise and help identifying and filling gaps in existing knowledge.

6.2 EU funded projects

The SCORE group´s hard work resulted in financial support by means of EU funded projects. With this financial support the network can ensure continuous research and expand their activities.

EU COST action entitled: "Sewage biomarker analysis for community health assessment" [ref. oc-2013-1-14763] (Network coordinator: Dr. Kevin Thomas, Norwegian Institute for Water Research (NIVA), Oslo, Norway)

Abstract of the project: "Sewage contains the excreted biomarkers of endogenous human metabolism that directly reflects the exposure and stressors placed upon an entire contributing community. The quantitative measurement of these specific biomarkers in sewage from communities allows the averaged patterns of factors related to lifestyle, disease and environment to be used for the assessment of community health. The Action will develop and expand an existing pan-European inter-disciplinary network, bringing together experts from relevant disciplines interested in the application and development of using the quantitative measurement of human biomarkers in sewage to evaluate lifestyle, health and exposure at the community level. In order to achieve its objectives the Action will manage a common Europe-wide testing platform that will develop best practice, provide a significant increase in the comparable spatio-temporal resolution of available data, coordinate the development of new biomarkers in sewage with focus on new psychoactive substances and new

biomarkers for the community assessment of factors such as environment, health, lifestyle and diet, and integrate sewage-based approaches with other available metrics. The Action will have a major impact on the development of this emerging field and ensure that the technology is used in a responsible and effective manner and its potentially fully exploited in collaboration with end-users".

The PhD candidate has been involved from the start and assisted in drafting the project. His role within the project consists on giving support to the coordinator of the project (Dr. Kevin Thomas) and the responsible of the UJI research group (Dr. Félix Hernández). He is member of the Management Committee (MC) and representative of Spain (*i.e.* the MC supervises and coordinates the COST Action). Furthermore he is, together with Prof. Dr. Pim de Voogt, co-leader of Work Group 1 "*WG1. Sewage biomarkers analysis: methods and technology*"

EU Marie Curie Initial Training Network, SEWPROF, entitled 'A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level' from the People Programme (Marie Curie Actions) of the European Union's 7th Framework Programme FP7/2007 - 2013/under REA grant agreement n° [317205]. (Network coordinator: Dr. Barbara Kasprzyk-Hordern, University of Bath, UK)

Abstract of the project: "SEWPROF aims to develop inter-disciplinary and cross-sectoral research capability for the next generation of scientists working in the newly-emerging field of sewage epidemiology. It will provide an integrated approach towards public health monitoring at a community level based on innovative sewage epidemiology techniques. The approach will deliver real-time profiling of community-wide health and lifestyle through the analysis of human biomarkers in sewage using a wide-range of methods including hyphenated mass spectrometry techniques, bioanalytical techniques and real-time sensing. The innovative research strategy of obtaining epidemiological information from sewage has been pioneered by members of the SEWPROF team, and, although still in its infancy, is currently used to determine illicit drug use trends at community level via the analysis of urinary biomarkers in sewage. SEWPROF aims to advance knowledge of the epidemiology of (illicit) drug use and to bridge gaps in the available expertise with the ultimate goal of applying this cutting edge interdisciplinary approach within epidemiological studies of societal health. This conceptually simple but methodologically sophisticated epidemiological approach could become an early warning system for outbreaks of disease and a unique tool for the identification of hot-spots for pandemics. This will be achieved through bringing together leaders in the field across Europe in academia, research institutes and the private sector and utilising their expertise and commitment in training a highly employable cohort of researchers who will acquire advanced knowledge in the field

through an interdisciplinary and cross-sectoral training programme delivered by world class research-led organisations and industrial partners".

Role of the PhD candidate within the project consists on giving support to the training leader of the project and responsible of the UJI research group, Prof. Dr. Félix Hernández. The PhD candidate has been involved and contributed to the project from the early beginning. He is attending the meetings of the network as a member of the UJI together with Dr. Hernández, and he will be the UJI representative in the case that Dr. Hernández cannot attend any of these meetings. He has also given some lectures during the training courses and coordinates the development of a database on drugs and metabolites on which the project focuses on.

6.3 Scientific articles

As outlined above, under the leading of SCORE group (formed by seven research centers/institutions) there has been an extensive collaboration within a network involving up to 20 research groups. This European collaboration has resulted in three joint peer-reviewed articles. The writing of these scientific articles was performed and coordinated by the SCORE group:

- *Science of the Total Environment* 432, **2012**, 432 – 439 (Thomas *et al.* 2012 - *scientific article a*)
- *Environmental Science and Technology* 47, **2013**, 1452 – 1460 (Castiglioni *et al.* 2013 - *scientific article b*)
- *Addiction* 109, **2014**, *1338 - 1352* (Ort *et al.* 2014 - *scientific article c*)

It is worth to notice that *scientific article a,* has recently been classified as top/highly cited paper by ISI Web of Knowledge (45 time cited, Scopus (June 2014)).

In addition, to these three scientific articles, a chapter has been submitted on invitation by the EMCDDA, for a monograph on SBE (*scientific monograph, chapter 2)*. This report is an update on a previously published monograph "EMCDDA insight 9: Assessing illicit drugs in wastewater: Potential and limitation of a new monitoring approach".

The contributions of the PhD candidate within SCORE and these publications mainly involved analytical-related issues, particulary in relation to LOQs reported by the different groups, LC and MS conditions employed by the laboratories, and the quality criteria applied. Furthermore, he helped to design the study and drafting the agreed protocol. Coordinated sampling and analysis in Castellón (Spain), Berlin, Dresden, Dülmen, Dortmund, and Munich (Germany), employing the validated analytical methodologies discussed in Chapter 2 of this thesis (Bijlsma *et al.* 2009, modified later by Bijlsma *et al.* 2014). He compiled and evaluated data, drafted and revised the manuscripts. Moreover, he attended meetings, workshops and conferences and assisted in writing workshop reports.

Chapter 6.3.1, scientific article a

Comparing illicit drug use in 19 European cities through sewage analysis

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Highlights

- First Europe-wide study of illicit drug use through sewage biomarker analysis
- First application of a harmonized protocol to report and evaluate sampling, analysis and data handling
- First inter-laboratory comparison of the analysis of illicit drugs
- Comparable illicit drug use data for 19 European cities
- Extrapolated total daily use of cocaine in Europe during the study period was equivalent to 356 kg/day

Abstract

The analysis of sewage for urinary biomarkers of illicit drugs is a promising and complementary approach for estimating the use of these substances in the general population. For the first time, this approach was simultaneously applied in 19 European cities, making it possible to directly compare illicit drug loads in Europe over a 1-week period. An inter-laboratory comparison study was performed to evaluate the analytical performance of the participating laboratories. Raw 24-hour composite sewage samples were collected from 19 European cities during a single week in March 2011 and analyzed for the urinary biomarkers of cocaine, amphetamine, ecstasy, methamphetamine and cannabis using in-house optimized and validated analytical methods. The load of each substance used in each city was back-calculated from the measured concentrations. The data show distinct temporal and spatial patterns in drug use across Europe. Cocaine use was higher in Western and Central Europe and lower in Northern and Eastern Europe. The extrapolated total daily use of cocaine in Europe during the study period was equivalent to 356 kg/day. High per capita ecstasy loads were observed in Dutch cities, as well as in Antwerp and London. In general, cocaine and ecstasy loads were significantly elevated during the weekend compared to weekdays. Per-capita loads of methamphetamine were highest in Helsinki and Turku, Oslo and Budweis, while the per capita loads of cannabis were similar throughout Europe. This study shows that a standardized analysis for illicit drug urinary biomarkers in sewage can be applied to estimate and compare the use of these substances at local and international scales. This approach has the potential to deliver important information on drug markets (supply indicator).

Keywords

Sewage biomarker analysis, Cocaine, Methamphetamine, Amphetamine, MDMA, *Cannabis*

1. Introduction

Illicit drug use and trafficking are international issues that have negative impacts across the social and economic spectrum, from the public health of individuals to the large-scale stability of national borders. Statistics show that around a third of European citizens have tried an illicit drug, while overdose claims the life of at least one citizen every hour (European Monitoring Centre for Drugs and Drug Addiction, 2010a). Ever-changing patterns in illicit drug production, demand and supply necessitate a program of frequent monitoring. Independent, objective and timely information on the type, scale and demographics of illicit drug use is essential in order to fully understand drug use and develop better methods and actions to respond to them. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) is responsible for collating such information in Europe and providing these data to policy makers so that they can design management strategies and appropriate laws. This information is also vital for measuring the success of existing management strategies and laws in view of a dynamic drug culture and emerging trends.

Illicit drug use is a socially stigmatized and often hidden activity, so traditional survey methods such as general population surveys and interviews can be inaccurate and prone to conjecture. The possibility of adding a new technique that overcomes these challenges to the existing repertoire of research methods is therefore an exciting prospect (European Monitoring Centre for Drugs and Drug Addiction, 2008). On average between 80 and 90% of the population is connected to the sewer network with much lower rates (40–65%) connected to primary sewage treatment in Eastern Europe. Sampling sewage at the inlet of a sewage treatment plant (STP) can therefore provide a diluted and pooled community urine sample that can deliver objective near real-time estimates of the total quantities of illicit drugs being used by the connected population (Zuccato et al., 2008, van Nuijs et al., 2011a and Daughton, 2011) (**Fig. S1**). The quantitative analysis of sewage for the estimation of illicit drug use is complementary to existing epidemiologically based approaches and can provide additional, evidence-based information. The potential of this approach to provide information regarding drug use at local level has previously been shown by Zuccato et

al. (2008) where reproducible and characteristic profiles of illicit drug use were obtained in three cities, quickly revealing any short-time changes. Subsequently this approach has been used to estimate local (i.e. city or small town) and national use (van Nuijs et al., 2011b), monitor use trends with time (short-term and long-term; Harman et al., 2011), identify changing trends and new habits (Reid et al., 2011a and Castiglioni et al., 2011a) and identify the use of new substances (Zuccato et al., 2008, van Nuijs et al., 2011a and Daughton, 2011). The conceptual advantages of estimating illicit drug use based on sewage analysis are that it provides an aggregated estimate of all the people contributing to the sewage in a catchment over the sampling period, is non-intrusive and ethical approval from individuals is of no concern, it is objective and does not have the problems of surveys that may suffer from a limited number of subjects and self-reporting bias, and the results can be obtained almost in real-time, which means within days or weeks compared to surveys that may take years before publication.

The approach has been applied in Australia, Europe, and North America yielding promising results (van Nuijs et al., 2011a, van Nuijs et al., 2011b, Daughton, 2011, Reid et al., 2011a, Castiglioni et al., 2011a and Irvine et al., 2011). However, coordinated international studies have yet to be performed, and the direct comparison of these data is not trivial. Challenges arise from uncertainties associated with the sampling of sewage, behavior of the selected biomarkers in the sewer, reliability of inter-laboratory analytical measurements, different back-calculation methods, and different approaches to estimate the size of the population being tested.

The objective of the present study was for the first time to apply sewage analysis simultaneously in 19 European cities over a single week, following a harmonized protocol to report and evaluate sampling, analysis and data handling. In this way, it was possible to compare patterns of illicit drug use across Europe in a sound and reliable way. Sewage samples were analyzed for biomarkers of cocaine (COC), amphetamine (AMP), methamphetamine (METH), ecstasy (as 3,4-methylenedioxymethamphetamine, MDMA), and cannabis (CAN). To evaluate the analytical performance of each participating laboratory, an inter-laboratory comparison exercise was performed. The resulting estimates of illicit drug use were then

further compared with official national statistics as compiled and reported by the EMCDDA (European Monitoring Centre for Drugs and Drug Addiction, 2010a).

The approach applied on a Europe-wide scale can reveal if trends of drug consumption can be promptly monitored at an international scale, using a tool which is complementary to more focused survey methods, and which has the potential to become an additional source of real-time epidemiological information.

2. Methods

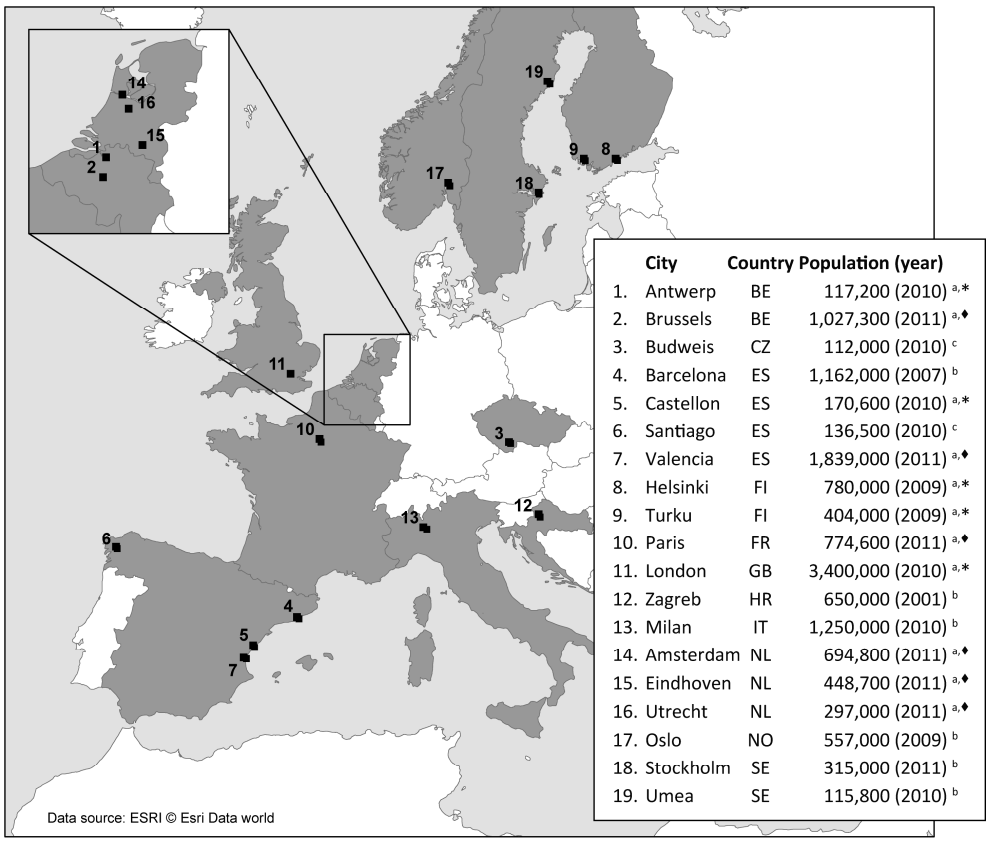
2.1. Sewer system characterization (questionnaire)

To assess the potential of sewage drug testing under normal operating conditions, we purposely relied on existing sampling procedures at the individual STPs. It is worth noting that the catchments and sewer systems cannot be controlled by the investigators and that both sewage flows and biomarker concentrations vary during a day; not only at the time-scales of hours but also minutes (Ort et al., 2010a). This has been neglected in most previous studies and relevant information, to evaluate the data quality from routinely collected sewage samples, has not been reported (Ort et al., 2010b). Therefore, we developed a specifically tailored questionnaire. In close cooperation with local sewer and STP operators it allowed project partners to gather important catchment and sewer system characteristics in a formalized manner. Information such as the presence and operation of lift stations (generating short hydraulic pulses), structural state of sewers (loss of sewage via exfiltration), variability of population size (commuters) and details on sampling procedures was considered to facilitate a meaningful data quality evaluation. Data were flagged when either excessive sewage leakage was reported or the sampling uncertainty was larger than inter-laboratory analytical variation.

2.2. Sampling

Raw sewage was collected from the inlet of 21 STPs spread over 11 European countries, representing nineteen cities (Valencia is served by three STPs), and servicing a combined population of approximately 15 million inhabitants (**Fig. 1**). Samples were collected from each location over seven consecutive days, starting on Wednesday 9th

March 2011 and ending on Tuesday 15th March 2011. In five STPs, interruptions due to technical problems caused smaller sample sizes. In Barcelona, the sampling campaign was performed one week later, between the 16th and 22nd March 2011.



	City	Country	Population (year)
1.	Antwerp	BE	117,200 (2010) a,*
2.	Brussels	BE	1,027,300 (2011) a,♦
3.	Budweis	CZ	112,000 (2010) c
4.	Barcelona	ES	1,162,000 (2007) b
5.	Castellon	ES	170,600 (2010) a,*
6.	Santiago	ES	136,500 (2010) c
7.	Valencia	ES	1,839,000 (2011) a,♦
8.	Helsinki	FI	780,000 (2009) a,*
9.	Turku	FI	404,000 (2009) a,*
10.	Paris	FR	774,600 (2011) a,♦
11.	London	GB	3,400,000 (2010) a,*
12.	Zagreb	HR	650,000 (2001) b
13.	Milan	IT	1,250,000 (2010) b
14.	Amsterdam	NL	694,800 (2011) a,♦
15.	Eindhoven	NL	448,700 (2011) a,♦
16.	Utrecht	NL	297,000 (2011) a,♦
17.	Oslo	NO	557,000 (2009) b
18.	Stockholm	SE	315,000 (2011) b
19.	Umea	SE	115,800 (2010) b

Figure 1. Cities that participated in the study. Population estimates in the catchment of the corresponding sewage treatment plants are based on the method that was deemed the most appropriate by the sewage treatment plant operators (see footnotes and text for more details). Population estimated based on: [a]Measured influent load: *BOD/COD/N/P (average of year calculated by sewage treatment plants operators), *BOD (7-day average during sampling week, 60 g BOD person^{-1} day^{-1}). [b]Census data. [c]Number of house connections or drinking water subscribers.

Samples were collected using the operational equipment of the individual STP. At all locations, 24-h composite samples were collected, while mode of sampling was either time-, volume- or flow-proportional (Ort et al., 2010a). Automated equipment was used at all locations with the exception of Barcelona. All samples were stored in silanized glass, polyethylene terephthalate (PET) or high-density polyethelene (HDPE) containers and analyzed within 12 h of collection. If this was not possible, the samples were immediately frozen at − 20 °C until analysis to prevent degradation of the illicit drug target residues. Based on published data on analyte stability there would not be a substantial difference between the two sample storage scenarios (i.e. 4 °C for 12 h or longer at − 20 °C) (Baker and Kasprzyk-Hordern, 2011 and Reid et al., 2011b). For each sample, the flow rate (L/day) of the sewage stream was recorded and where possible, water quality parameters such as total nitrogen (N), total phosphorus (P), chemical oxygen demand (COD) and biological oxygen demand (BOD) were measured in order to support estimating the contributing population and normalize for it (Andreottola et al., 1994, Zessner and Lindtner, 2005 and Garnier et al., 2006).

2.3. Analysis

2.3.1. Analytical methodology

The physical and chemical composition of sewage is rather complex and includes large loadings of suspended particulates as well as the presence of relatively high concentrations of compounds that can potentially interfere with the analysis of the target drugs. Therefore the sewage samples were filtered (filter type GFC, 0.45 μm), concentrated and cleaned-up using solid phase extraction (SPE) prior to analysis. All participants used SPE for the preconcentration of samples typically using polymeric cartridges (e.g. Oasis HLB) in off-line or on-line mode (Table S1). More details on analytical methodology can be found elsewhere (Castiglioni et al., 2006; Kasprzyk-Hordern et al., 2008; Postigo et al., 2008; Bijlsma et al., 2009; Hogenboom et al., 2009; van Nuijs et al., 2009; Terzic et al., 2010; Vazquez-Roig et al., 2010; Reid et al., 2011a; González-Mariño et al., 2012). The analytical technique of choice for the quantitative analysis of the target drug residues was liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Most participating groups used triple quadrupole or hybrid quadrupole-linear ion trap mass analyzers, with the exception of KWR (The

Netherlands) and the University of Santiago de Compostela (Spain) who used high-resolution mass spectrometry (LTQ-Orbitrap and time-of-flight MS, respectively).

These highly sensitive analyzers, together with steps to concentrate and clean-up the sewage samples enable analysis at a low concentration level (ng/L) in sewage (van Nuijs et al., 2011a and Castiglioni et al., 2011a). The use of mass labeled internal standards is mandatory to compensate for the potential analytical errors associated with the sample manipulation and due to matrix effects (increase or decrease of analytical signal due to matrix interferences). Internal standards were added to the samples prior to sample treatment. Each participating laboratory used an in-house and fully validated analytical method. The methods comply with identification and confirmation criteria (The Commission of the European Communities, 2002) used in this type of LC–MS/MS analysis to avoid the reporting of false positives or negatives. The analytical quality assurance was evaluated by an inter-laboratory study of two external quality control solutions (50 ng/mL and 500 ng/mL). The mean concentrations reported by participating laboratories (n = 11, except for cocaine, n = 12, and THC n = 9) varied between 49 and 60 ng/mL for the low-level solution and between 460 and 530 ng/mL for the high-level solution. Inter-laboratory variation (relative standard deviation (RSD)) varied between 26 and 38% for the low-level spike and between 12 and 26% for the high-level spike. With regard to the sigma difference of the two standard solutions (450 ng/mL), the average recovery of the spike in both samples ranged between 91 and 111% for all laboratories. The recovery of the spike, the systematic errors of the measurements and the between-laboratory systematic errors represent aspects of accuracy. The precision is represented by the coefficient of variation of reproducibility and the outliers of the sigma difference. Combining the accuracy and precision, a report mark per parameter was awarded. The average report mark was 8.3 out of 10 (lowest for amphetamine 7.0 and the highest for MDMA and benzoylecgonine 9.0).

2.3.2. Normalization and estimation of use

The use of the main classes of illicit drugs was assessed by measuring specific target biomarkers in urban sewage. The main requirements for the selection of a target residue were the presence in sewage at quantifiable concentrations, and the stability of the

target residue from chemical and biological degradation in the sewer and during sampling and storage. Parent drugs were chosen as target residues for amphetamine-type stimulants (AMP, METH, and MDMA), while the main urinary metabolites were chosen for cocaine (COC) and cannabis (CAN), which were benzoylecgonine (BE) and 11-nor-9-carboxy-delta9-tetrahydrocannabinol (THC-COOH) respectively (Zuccato et al., 2008 and van Nuijs et al., 2011a). The amount (daily mass load) of each target residue that was excreted by a population was calculated by multiplying measured sewage concentrations (ng/L) by corresponding daily flow rates of sewage (L/day) during the sampling campaigns. Data were then normalized by population size (1000 inhabitants) to allow comparison among different cities. Different approaches were used to estimate the population served by each STP due to the specific characteristics of the individual sewer systems investigated (i.e. census data, number of house connections or drinking water subscribers, or using a measured biological parameter in sewage; BOD, COD, N, and P; Andreottola et al., 1994, Zessner and Lindtner, 2005 and Daughton, 2012). The variability among the different population estimates obtained was found to be between 7 and 40% (%RSD) and is strictly dependent on the sewage composition (i.e. presence of industrial wastes which can increase the hydrochemical parameters) or the sewer system characteristics. It was therefore not possible to use a unique approach to estimate the population at all sites, and the most reliable estimation was selected on a case by case basis based upon the expert knowledge of the local STP operators.

The daily loads relative to population (mg/day/1000 inhabitants) calculated for target residues of AMP, METH, MDMA, and CAN were averaged over all sampling days to compare illicit drug use across the investigated cities. For COC, it was possible to back-calculate its consumption from the measured normalized daily loads of BE using the model suggested by Zuccato et al. (2008), which contains parameters for human metabolism and the subsequent excretion of BE as well as the molar ratio of COC/BE. A complete review of all COC pharmacokinetic studies available in the literature suggests a median excretion value of 38% of a cocaine dose and a correction factor of 2.77 of BE excretion (Castiglioni et al., 2011b).

3. Results

3.1. System characterization and sampling

The questionnaire had a very good response and all but one of the operators were very cooperative. The results show that 10 of 20 STPs sample volume proportionally with average sampling intervals of around 15 min, which resulted in generally acceptable uncertainties with only two potentially critical sites. Similarly, flow data quality also seems satisfactory since 10 of the 20 STPs reported that they calibrate their flow meters in periods of between 1 month and 6 years. Systematic flow measurement errors cancel out when population size is determined from wastewater parameters (e.g. BOD); i.e. if a too high illicit drug load was calculated due to a too high flow, this would similarly hold true for the BOD load resulting in an overestimation of the population directly proportional to the overestimation of illicit drug loads (Lai et al., 2011). Only for five STPs, for which the served population was not determined by means of a wastewater parameter, was the flow meter calibration not reported. Uncertainties of daily flow were reported to be between 0.3 and 8% (median: 1%), which are rather low. Sewage losses were reported for four catchments; in two cases, a sewage loss of > 20% may also imply a biomarker load loss of > 20% (**Fig. 2**).

3.2. Patterns of illicit drug use in Europe

3.2.1. Cocaine (COC)

The COC use estimates (averaged for all sampling days), calculated from BE loads, are shown in **Fig. 2A**. COC use could be estimated for all investigated cities, since BE could always be quantified in sewage. The highest use of COC was observed in Antwerp followed by (in order of decreasing use) Amsterdam, Valencia, Eindhoven, Barcelona, London, Castellón, and Utrecht, where the average estimates were in the range of 987–1998 mg/day/1000 inhabitants. Milan, Santiago de Compostela, Paris, and Brussels showed estimates of COC use around 511–662 mg/day/1000 inhabitants, while the average estimates were lower in Budweis, Zagreb, Helsinki, Turku, Oslo, Stockholm, and Umeå, between 2 and 146 mg/day/1000 inhabitants.

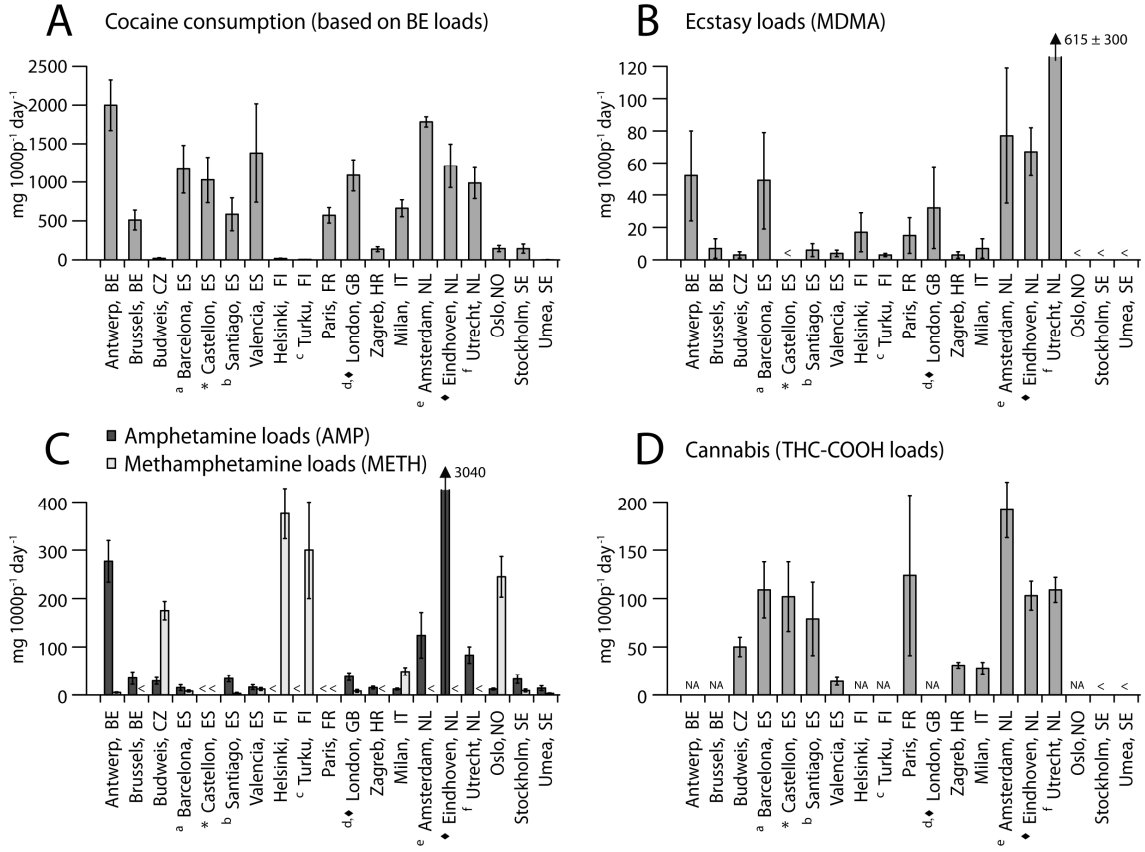


Figure 2. Average estimates of cocaine (COC) consumption (back-calculated from benzoylecgonine (BE) loads, the main metabolite of COC) and population-normalized loads of amphetamine (AMP), methamphetamine (METH) in 19 selected European cities and cannabis (THC-COOH, all in mg/1000 inhabitants/day) in 13 of them between the 9th and 15th March 2011 (mean ± SD from all sampling days, n = 7). [a]Sampling one week later (16th–22th March 2011). [b]n = 6, sample of Monday 14th March missing. [c]n = 6, sample of Sunday 13th March missing. [d]n = 6, sample of Saturday 12th March missing. [e]n = 6, sample of Monday 14th and Tuesday 15th March missing. [f]Sampling started one day later (10th–16th March 2011). *Sampling uncertainty estimated to be larger than variation of interlaboratory comparison of chemical analysis. ◆Exfiltration of sewage larger than 20%. [NA]Not analyzed. [<]All measured concentrations were below the limit of quantification.

3.2.2. Ecstacy (MDMA)

Results of the MDMA analysis are presented in **Fig. 2B**. The three Dutch cities (Amsterdam, Utrecht, and Eindhoven) showed the highest loads of MDMA relative to population size with average loads of between 67 and 615 mg MDMA/day/1000 inhabitants. Results from Antwerp and London also indicate large quantities of MDMA relative to population size (32–52 mg MDMA/day/1000 inhabitants). MDMA was not detected in Castellon, Umeå, Stockholm, and Oslo.

3.2.3. Amphetamines (AMP and METH)

METH was detected in the majority of sewage samples with the exception of Zagreb, Paris, and Castellón and the three cities included from the Netherlands (Amsterdam, Eindhoven and Utrecht). Measurements in both Helsinki and Turku showed the highest METH loads relative to population size with 376 and 300 mg METH/day/1000 inhabitants, respectively (**Fig. 2C**). Results from Oslo (245 mg METH/day/1000 inhabitants) and Budweis (175 mg METH/day/1000 inhabitants) also indicate large quantities of METH relative to their population size. The remaining cities show relatively low METH loads relative to population size (3–49 mg METH/day/1000 inhabitants). It should be noted that in the cities with relatively low METH use, relatively high use of AMP was observed, and vice versa.

AMP was the dominant amphetamine-like drug in Zagreb, Valencia, Barcelona, Castellón, Santiago de Compostela, Stockholm, Umeå, and London. Most notable, however, were the results from Belgium and The Netherlands with 33–3040 mg AMP/day/1000 inhabitants. The result from Eindhoven is extremely high in relation to all other cities, and is, in fact, the highest result ever recorded in the Netherlands.

3.2.4. Cannabis (THC-COOH)

The highest measured per-capita loads of THC-COOH (192 mg THC-COOH/day/1000 inhabitants) were observed in Amsterdam (**Fig. 2D**). Results for other cities were in the range of 14–124 mg THC-COOH/day/1000 inhabitants. The analysis of THC-COOH in sewage was not performed for Antwerp, Brussels, London, Oslo, Helsinki and Turku, due

to validated methods not being available in all participating laboratories, and it was below the detection limit in Umeå and Stockholm.

3.3. Weekly patterns in illicit drug use

The day-to-day variation of illicit drug loads is charted in **Fig. 3** for COC and MDMA as percentage of the measured total weekly load. The MDMA loads for Utrecht were excluded from the analysis since it is possible that they are related to the dismantling of an illegal production facility. Besides Utrecht the following cities were also excluded from the analysis of weekly MDMA pattern since all values were below the limit of quantification: Oslo, Stockholm, Umeå and Castellón. Cocaine use (based on BE loads) and MDMA loads show a clear weekly pattern characterized by an increase of use during the weekend compared to weekdays: 14 out of 19 cities had highest COC use on Saturday or Sunday. The increased BE loads on Saturday and Sunday indicate elevated COC use on Friday and Saturday nights (including the early hours of Sunday morning). The use over all cities on an individual weekday (MON-FRI) was approx. 13% (median) of the total weekly use, increasing significantly to 18% on a weekend-day (SAT/SUN): p = 1.2e-6, Wilcoxon test *weekend-day* (*SAT/SUN*) vs. *weekday* (*MON–FRI*). If the weekend was extended and includes Friday the effect is still significant, but slightly weaker: p = 3.2e-6, Wilcoxon test *weekend-day* (*FRI–SUN*) vs. *weekday* (*MON–THU*). Median MDMA loads more than doubled on a weekend-day compared to a weekday, from 10% (MON–FRI) to 24% (SAT/SUN) of the total weekly load: p = 1.3e-8, Wilcoxon test *weekend* (*SAT/SUN*) vs. *weekdays* (*MON–FRI*). Even if Sunday alone was tested against all other days as weekdays (MON–SAT) the increase is still significant: p = 1.6e-5, Wilcoxon test *SUN* vs. *weekdays* (*MON–SAT*).

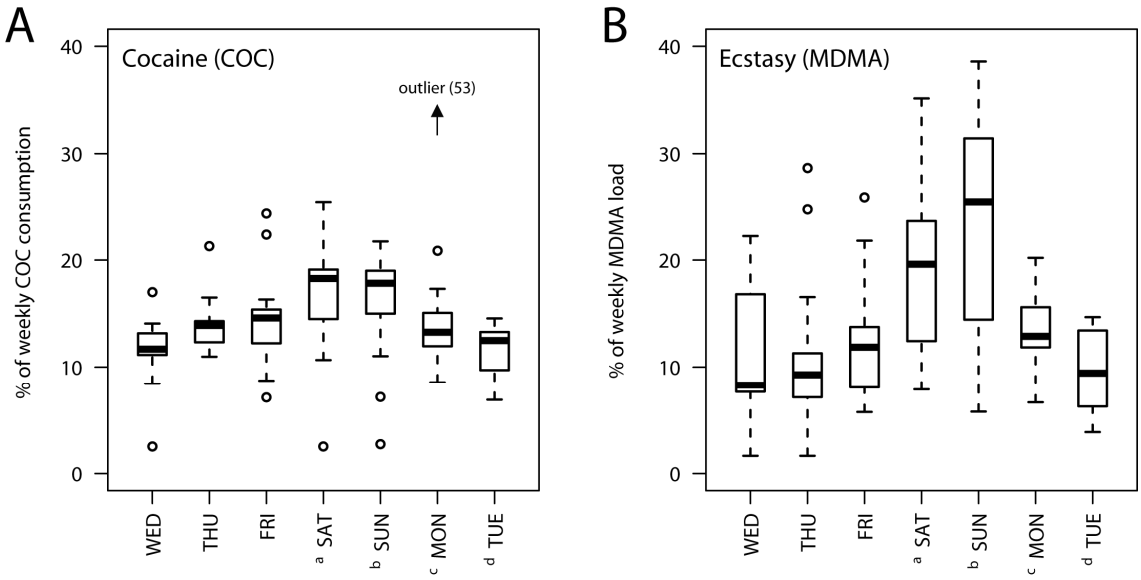


Figure 3. Day-to-day variation of cocaine (COC) consumption (based on the loads of the main metabolite benzoylecgonine (BE)) in 19 cities and ecstasy (MDMA) in 15 cities. Medians are significantly different on the weekend compared to weekdays. [a]Sample from London missing (COC n = 18, MDMA n = 14). [b]Sample from Turku missing (COC n = 18, MDMA n = 14). [c]Sample from Amsterdam and Santiago missing (COC n = 17, MDMA n = 13). [d]Sample from Amsterdam missing (COC n = 18, MDMA n = 14).

4. Discussion

4.1. System characterization and sampling

Our results show that a thorough analysis of sewage systems, which requires close collaboration with local STP operators, and sampling design are necessary to obtain good, reliable data. The results of this study revealed only a few cases where the level of confidence associated with the STPs' own sampling equipment and protocol was unacceptable. This supports the recent findings of Mathieu et al. (2011) who suggest that the STPs' own sampling equipment is usually suitable for this type of study where the catchments houses more than 100,000 inhabitants. Given the observed variability across Europe (**Fig. 2**), an estimated additional variability of 10–30% from discharge

measurements would not affect the general conclusions of this study. The consequences of sewage losses, for example from re-use or sewer leakage are generally difficult to assess and need to be factored in on a case by case basis.

4.2. Cocaine

COC use estimates in 19 European cities were in the range of 2–1998 mg COC/day/1000 inhabitants (**Fig. 2A**). A notable trend was that COC use is higher in more urbanized towns/cities within the same country. For example, COC use in the Swedish town of Umeå (population 75,000) resulted in population-normalized loads of 2 mg COC/day/1000 inhabitants, while results from the Swedish capital of Stockholm (population 850,000) were substantially higher at 145 mg COC/day/1000 inhabitants. The same trend was observed in Finland where the smaller city of Turku (6 mg COC/day/1000 inhabitants) showed much lower population-normalized loads than the capital Helsinki (18 mg COC/day/1000 inhabitants). Similar patterns have been observed in The United States with higher COC use in the more urbanized cities (Banta-Green et al., 2009).

Any comparison between national annual prevalence data and a city-specific, one-week snapshot based on sewage analysis, must be treated with caution due to intra-country temporal and spatial variability. However, a general assessment as to whether the sewage-based data reflect the known information obtained via socio-epidemiological surveys is of value. The highest European prevalence of COC use among the general population (aged 15–64) and young adults (aged 15–34) is reported to be in Spain, the United Kingdom, Italy, Ireland and Denmark (European Monitoring Centre for Drugs and Drug Addiction, 2010a). Among the countries included in our study, the lowest prevalence data are reported for Norway, Sweden, Finland, the Czech Republic, France and the Netherlands, with no data available for Belgium and Croatia. The COC use estimates based on sewage analysis in general agree with these data, except for a few cases (**Fig. 2**). Sewage analysis found that COC use in Dutch cities is higher than would be expected from the 2010 prevalence data. Furthermore, prevalence data for Italy are among the highest in Europe, while estimates for Milan from sewage analysis show lower than expected use (**Fig. 2**). A possible explanation for

this discrepancy may be the reduction in COC use in Italy, which has been observed between 2008 and 2009 through sewage analysis (Zuccato et al., 2011), and subsequently confirmed in 2010 by national epidemiological surveys (Dipartimento Politiche Antidroga, 2010).

There is clearly a need to better understand how much COC is being used in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2010a) and sewage analysis can be applied as a supply indicator to assess the size of the European consumer market for illicit drugs. Using the mean daily COC estimate (708 mg/day/1000 inhabitants) and a total European population of 502 million people, it appears that during the study period, approximately 355 kg/day of pure cocaine was used in Europe. It is clear that this is only a crude estimate, based upon the testing of only 2% of the population from a selection of cities and countries over a limited period. Further refinement of the calculation methodology and the wider application of the technique, possibly on a country basis, would provide a valuable insight into the amounts of COC used across Europe.

4.3. MDMA

The Czech Republic and the United Kingdom are reported to have the highest prevalence of ecstasy use among the general population (aged 15–64). In the young adults group (aged 15–34), the Netherlands is also reported to be a country with a high prevalence of MDMA use (European Monitoring Centre for Drugs and Drug Addiction, 2010a). Additionally, as already discussed in the previous paragraph, Belgium and the Netherlands are reported to be the main countries where MDMA is produced. Sewage analysis results from the present study reflect the published prevalence data with high per capita loads detected in Eindhoven, Amsterdam and London. The per capita MDMA loads detected in Utrecht were substantially higher than any other European city and more than 10 times higher than previously measured in Utrecht (Bijlsma, pers. comm.). It is possible that a police raid on an illegal MDMA manufacturing facility in Utrecht two days prior to the study resulted in MDMA being released into the city's sewer network resulting in the high levels that were measured. If this is the case, then the signal does not reflect the actual use of MDMA in Utrecht during this study.

Other high per capita MDMA loads were detected in Antwerp, situated close to the Dutch border, and in Barcelona, compared with other locations sampled in both countries. The fact that MDMA was not detected in Swedish sewage is in agreement with MDMA prevalence data, which states that Sweden is a low-prevalence country, along with the other Nordic countries of Norway and Finland. The discrepancy between the low levels of MDMA detected in Czech sewage and the reported highest national prevalence of ecstasy use in Europe may possibly be explained by the local situation that may not be representative of the Czech Republic. The MDMA loads measured in the present study compared well with previous European reports using the sewage analysis approach, both in terms of extent of the average load and of weekly variation profile (van Nuijs et al., 2011a). As observed for COC and MDMA, in the majority of cases the use was higher in highly populated urban centers.

4.4. Amphetamines

The results from sewage analysis indicate that Finland, Norway and the Czech Republic have the highest rates of METH use per-capita. These results correspond well with reported statistics that highlight the significant amount of METH that is regularly seized in the Nordic region and North East Europe (European Monitoring Centre for Drugs and Drug Addiction, 2010a). Interestingly, the same three nations have notably low rates of COC use (**Fig. 2**), which may indicate an inverse relationship between the two drugs. One simple explanation may be price and availability, whereby the use of COC is diminished in the face of a readily available supply of the substantially cheaper drug METH. However, Finland, Norway and the Czech Republic represent only a small fraction of the total European population.

Our results indicate that AMP dominates the amphetamine-like drug class on a European-wide perspective with respect to per-capita drug levels in sewage. Of particular interest are the per-capita loads of AMP in the cities of Northern Belgium (Antwerp) and the Netherlands which are the highest in Europe. These results are in contrast to the published drug-use prevalence data which suggest that the use of AMP in these nations is actually a factor of 2–3 lower than in the rest of Europe. One possible explanation is that the Netherlands, Poland and Belgium are major producers of AMP

(European Monitoring Centre for Drugs and Drug Addiction, 2010b). In 2008, 38% of the sites identified in the EU member states as being involved in production of AMP were discovered in the Netherlands. This is important for the interpretation of sewage analysis results, since the AMP and METH loads in these countries may reflect both the use of these substances and also release from drug production facilities. AMP and METH can also arise in sewage systems from the metabolism of prescribed medicines (e.g. Selegiline) which may add to the signal being measured, however prescription rates suggest that any contribution would be < 1% of the total amphetamine signal.

4.5. Cannabis

During 2010, cannabis was used by around 7% of the population aged between 15 and 64 making it the most popular drug in Europe (European Monitoring Centre for Drugs and Drug Addiction, 2010a). In the smaller cities of the Netherlands (Eindhoven and Utrecht) comparably high loads of cannabis were found in the sewage with a maximum observed in Amsterdam. This may be explained by the liberal Dutch drug policy on cannabis use, which is permitted for every citizen over the age of 18 years. However, the prevalence of cannabis use is reported to be greatest in the Czech Republic, Italy, Spain and France. Spain, due to its strategic trafficking location, is an important cannabis market, and besides Barcelona, the smaller cities of Castellón and Santiago de Compostela also show high rates of use. Yet, per capita THC loads measured in Valencia are much lower than would be expected from published prevalence data. In Milan, the determined loads are also relatively low, and not in line with the cannabis use in Italy reported by the monitoring authorities. When interpreting these results the THC content of resin and herbal cannabis should be considered, since it can vary strongly from country to country, ranging from 3 to 17% (resin cannabis) and from 1 to 15% (herbal cannabis).

4.6. Weekly patterns of drug use

Daily changes in illicit drug use were reflected in changes in STP influent sewage loads. The patterns in COC use showed clearly that there was increased use during the weekend compared to weekdays (**Fig. 3**), reflecting the recreational use of this drug. A trend in the MDMA loads along the week could also be observed, with statistically

higher loads during the weekend (Sunday). The higher ecstasy loads measured during the weekend are in agreement with the recreational character of this substance and its popularity in the dance and music scene. For AMP, METH and THC-COOH, no weekly patterns in their loads could be observed, which is in agreement with the known pattern of use of these substances.

5. Conclusions

For the first time sewage analysis using a uniform protocol has been simultaneously applied in 19 European cities to estimate and compare the use of illicit drugs across Europe. The quantitative, non-intrusive, objective and rapid analysis of the illicit drug use of 15 million individuals was determined over a 1-week period, providing the most current data on illicit drug use in Europe with the results generally being in good agreement with officially reported prevalence data. In addition the approach also has the potential to be used as a supply indicator of the international illicit drug market. The present study clearly reinforces the conceptual strengths of analyzing biomarkers in sewage to produce objective and updated data on the use of illicit drugs and their market at local, national and international scales.

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Supplementary information

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References

Andreottola G, Bonomo L, Poggiali L, Zaffaroni C. A Methodology for the Estimation of Unit Nutrient and Organic Loads from Domestic and Non-Domestic Sources. Europe Water Pollut Contam 1994; 4: 13 – 19.

Baker DG, Kasprzyk-Hordern, B. Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography–mass spectrometry. J. Chromatogr. A 2011; 1218(12): 8036 - 8059.

Banta-Green CJ, Field JA, Chiaia AC, Sudakin DL, Power L, de Montigny L. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymeth-amphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal sewage. Addiction 2009; 104: 1874 – 1880.

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

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

Castiglioni S, Bagnati R, Melis M, Panawennage D, Chiarelli P, Fanelli R, Zuccato E. Identification of cocaine and its metabolites in urban sewage and comparison with the human excretion profile in urine. Water Res 2011a; 45: 5141 – 5150.

Castiglioni S, Zuccato E, Fanelli R. Illicit Drugs in the Environment: Occurrence, Analysis, and Fate Using Mass Spectrometry. New Jersey: John Wiley & Sons Inc. 2011b.

Daughton CG. Illicit Drugs: Contaminants in the Environment and Utility in Forensic Epidemiology. Rev Environ Contam Toxicol 2011; 210: 59 – 110.

Daughton CG. Real-time estimation of small-area populations with human biomarkers in sewage. Sci Tot Environ 2012; 414: 6 – 21.

European Monitoring Centre for Drugs and Drug Addiction. Assessing illicit drugs in sewage - Potential and limitations of a new monitoring approach. Luxembourg: Office for Official Publications of the European Communities, 2008.

European Monitoring Centre for Drugs and Drug Addiction.. The state of the drugs problem in Europe. Luxembourg: Office for Official Publications of the European Communities. 2010a.

European Monitoring Centre for Drugs and Drug Addiction. 2010b. Problem amphetamine and methamphetamine use in Europe. Luxembourg: Office for Official Publications of the European Communities. 2010b.

Garnier J, Laroche L, Pinault S. Determining the domestic specific loads of two sewage plants of the Paris conurbation (France) with contrasted treatments: A step for exploring the effects of the application of the European Directive. Water Res 2006; 40: 3257 – 3266.

González-Mariño I, Quintana JB, Rodríguez I, González-Díez M, Cela R. Screening and Selective Quantification of Illicit Drugs in Wastewater by Mixed-Mode Solid-Phase Extraction and Quadrupole-Time-of-Flight Liquid Chromatography-Mass Spectrometry. Anal Chem 2012; 84: 1708 - 1717.

Harman C, Reid MJ, Thomas KV. In Situ Calibration of a Passive Sampling Device for Selected Illicit Drugs and Their Metabolites in Sewage, And Subsequent Year-Long Assessment of Community Drug Usage. Environ Sci Technol 2011; 45: 5676 – 5682.

Hogenboom AC, Van Leerdam JA, de Voogt P. Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography-LTQ FT Orbitrap mass spectrometry. *J Chromatogr A* 2009; 1216: 510 – 519.

Irvine RJ, Kostakis C, Felgate PD, Jaehne EJ, Chen C, White JM. Population drug use in Australia: A sewage analysis. Foren Sci Int 2011; 210: 69 - 73.

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ. Multiresidue methods for the analysis of pharmaceuticals, personal care products, and illicit drugs in surfacewater and wastewater by solid-phase extraction and ultra performance liquid chromatography-electrospray tandem mass spectrometry. Anal Bioanal Chem 2008; 391: 1293 – 1308.

Lai FY, Ort C, Gartner C, Carter S, Prichard J, Kirkbride P, Bruno R, Hall W, Eaglesham G, Mueller JF. Refining the estimation of illicit drug consumptions from wastewater analysis: co-analysis of prescription pharmaceuticals and uncertainty assessment. Wat Res 2011; 45: 4437 – 4448.

Mathieu C, Rieckermann J, Berset J-D, Schürch S, Brenneisen R. Assessment of total uncertainty in cocaine and benzoylecgonine sewage load measurements. Wat Res 2011; 45: 6650 – 6660.

Ort C, Lawrence MG, Rieckermann J, Joss A. Sampling for Pharmaceuticals and Personal Care Products (PPCPs) and Illicit Drugs in Sewage Systems: Are Your Conclusions Valid? A Critical Review. Environ Sci Technol 2010a; 44: 2495 – 2521.

Ort C, Lawrence MG, Reungoat J, Mueller JF. Sampling for PPCPs in sewage systems: Comparison of different sampling modes and optimization strategies. Environ Sci Technol 2010b; 44: 6289 – 6296.

Postigo C, Lopez de Alda MJ, Barceló, D. Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on - line solid - phase extraction-liquid chromatography - electrospray-tandem mass spectrometry. Anal Chem 2008; 80: 3123 – 3134.

Reid MJ, Langford KH, Mørland J, Thomas KV. Quantitative assessment of time dependent drug-use trends by the analysis of drugs and related metabolites in raw sewage. Drug Alcohol Depend 2011a; 19: 179 - 186.

Reid MK, Harman C, Grung M, Thomas KV. The current status of community drug testing via the analysis of drugs and drug metabolites in sewage. Norwegian Journal of Epidemiology 2011b; 21 (1): 15 - 23.

Relazione annuale al Parlamento sull'uso di sostanze stupefacenti e sulle tossicodipendenze in Italia [English: Annual report to Parliament on the use of drugs and drug addiction in Italy]. 2010. Dipartimento Politiche Antidroga, Presidenza del Consiglio dei Ministri, Rome, Italy, available http://www.politicheantidroga.it/media/333123/4%200.2%20sintesi_2010_23_06.pdf;

Terzic S, Senta I, Ahel M. Illicit drugs in wastewater of the city of Zagreb (Croatia) – estimation of drug abuse in a transition country. Environ Pollut 2010; 158: 2686 – 2693.

The Commission of the European Communities. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Commun. 2002;L221/8. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF; Accessed February 23, 2012.

van Nuijs A, Tarcomnicu I, Jorens PG, Bervoets L, Blust R, Neels H, Covaci A. Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 2009; 395: 819 – 828.

van Nuijs A, Castiglioni S, Tarcomnicu I, Postigo C, Lopez de Alda M, Neels H, Zuccato E, Barcelo D, Covaci A. Illicit drug consumption estimations derived from sewage analysis: A critical review. Sci Tot Environ 2011a; 409(19):3564 – 3577.

van Nuijs A, Mougel J-F, Tarcomnicu I, Bervoets L, Blust R, Jorens PG, Neels H, Covaci A. Sewage epidemiology — A real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium. Environ Int 2011; 37: 612 – 621.

Vazquez-Roig P, Andreu V, Blasco C, Picó Y. SPE and LC-MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (València, Spain). Anal Bioanal Chem 2010; 397: 2851 – 2864.

Zessner M, Lindtner S. Estimations of municipal point source pollution in the context of river basin management. Wat Sci Technol 2005; 52: 175 – 182.

Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R. Estimating Community Drug Use by Sewage Analysis. Environ Health Perspect 2008; 116: 1027 – 1032.

Zuccato E, Castiglioni S, Tettamanti M, Olandese R, Bagnati R, Melis M, Fanelli R. Changes in illicit drug consumption patterns in 2009 detected by sewage analysis. Drug Alcohol Depend 2011; 118: 464 – 469.

Chapter 6.3.2, scientific article b

Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers

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Evaluation of Uncertainties Associated with the Determination of Community Drug Use through the Measurement of Sewage Drug Biomarkers

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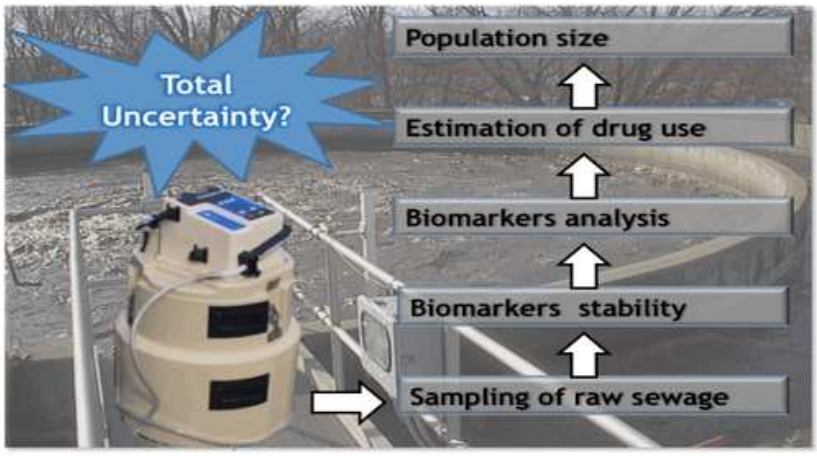
Graphical Abstract



Abstract

The aim of this study was to integrally address the uncertainty associated with all the steps used to estimate community drug consumption through the chemical analysis of sewage biomarkers of illicit drugs. Uncertainty has been evaluated for sampling, chemical analysis, stability of drug biomarkers in sewage, back-calculation of drug use (specific case of cocaine), and estimation of population size in a catchment using data collected from a recent Europe-wide investigation and from the available literature. The quality of sampling protocols and analytical measurements has been evaluated by analyzing standardized questionnaires collected from 19 sewage treatments plants (STPs) and the results of an interlaboratory study (ILS), respectively. Extensive reviews of the available literature have been used to evaluate stability of drug biomarkers in sewage and the uncertainty related to back-calculation of cocaine use. Different methods for estimating population size in a catchment have been compared and the variability among the collected data was very high (7–55%). A reasonable strategy to reduce uncertainty was therefore to choose the most reliable estimation case by case. In the other cases, the highest uncertainties are related to the analysis of sewage drug biomarkers (uncertainty as relative standard deviation; RSD: 6–26% from ILS) and to the back-calculation of cocaine use (uncertainty; RSD: 26%). Uncertainty can be kept below 10% in the remaining steps, if specific requirements outlined in this work are considered. For each step, a best practice protocol has been suggested and discussed to reduce and keep to a minimum the uncertainty of the entire procedure and to improve the reliability of the estimates of drug use.

1. Introduction

The chemical analysis of illicit drug excretion products in urban sewage collectively produced by a population has recently been established as a complementary approach for monitoring patterns and trends of illicit drug use in communities.[1, 2] The approach is based on the principle that the active parent compounds and/or metabolic residues of any substance ingested in the human body are excreted with urine into urban sewer networks. The concentrations of illicit drug metabolic residues measured in raw urban sewage can therefore reflect the amount of a particular drug that has been consumed by the population served by a sewer network.[3, 4]

The analysis of sewage drug biomarkers (SDBs) has been recently used to estimate illicit drug consumption at a community level in several cities worldwide.[1, 2, 5-12] The results derived from these studies generally show good agreement with prevalence data from national epidemiological surveys,[1, 13] demonstrating the potential of the approach to complement existing socio-epidemiological methods (e.g., population surveys, crime statistics, medical records, and seizure data). Although different from conventional methods for estimating drug use, the added value of the approach in generating useful data has recently been acknowledged. The European Monitoring Centre for Drug and Drug Addiction (EMCDDA) has shown interest in exploring the potential of wastewater analysis for enhancing drug monitoring in Europe by supporting a Europe-wide demonstration program in 2012.

The main advantage of sewage analysis for SDBs is to provide evidence-based, objective, and near-real-time estimates of illicit drug use in a defined population overcoming several of the limitations faced by conventional approaches such as the low objectivity of estimates, long study times, and high costs.[14] Recent studies demonstrate that the approach can be used not only to estimate consumption in a community (i.e., city, small town, schools, prisons),[1, 2, 4-13, 15] but also to identify changes during special events (i.e., sporting events, festivals, or holiday periods), to monitor changes in use with time, and to identify new habits or the use of new substances.[16-19] This has been made possible through the development of specific analytical

techniques that are able to detect trace quantities of SDBs in complex matrices, such as urban sewage.[2]

However, this approach is also subjective to a number of uncertainties associated with the different steps involved.[13] The main uncertainties are those related to the sampling of sewage, the stability of SDBs in sewage, the reliability of analytical measurements, the use of sound methods to back-calculate drug use, and the estimation of the size of the population under investigation. Some of these factors have recently been explored,[20, 21] and further research is still required in order to produce homogeneous data for the regional and international comparison of drug use estimates by sewage analysis.

The present study emerges within the framework of a recently performed Europe-wide investigation aimed at developing a common protocol to determine community drug use through the measurement of SDBs.[22] On the basis of the current understanding of best-practice, 14 participating research groups have agreed on a common protocol and uniform criteria regarding sample collection, storage, and analytical procedure. In this paper we attempt, for the first time, to integrally address the uncertainties associated with the estimation of community drug use through sewage analysis. All the critical steps were considered, and an original data elaboration based on an extensive review of the available knowledge for stability of SDBs in sewage and back-calculation of drug use was provided. The uncertainties related to sampling, chemical analysis, and estimation of population size in a catchment have been evaluated using the most extensive set of sampling and analytical data available at the moment and collected from 21 sewage treatment plants (STPs) in 19 European cities.

2. Experimental section

2.1. Sewer sampling and catchment characterics

Collection of important meta-data. Information on sampling protocols and the interpretation of sewage data for a given catchment is often missing or not reported.[23] Therefore, a standardized questionnaire was developed to systematically gather relevant information for each STP with two main purposes: (i) to characterize

catchments and monitoring periods—e.g., population size, exfiltration of wastewater, special events; and (ii) to assess the suitability of the sampling setup—e.g., flow variations, sampling mode, and frequency. The questionnaire is structured into 12 main sections (**Table S1** and **pdf file in SI**). The answers to 47 individual questions were classified into four categories according to their relevance for our aims. A meaningful evaluation was targeted with the availability of answers to the majority of category I questions. Sampling uncertainty was assessed by estimating the expected influent load variations with a stochastic model,[24] considering intraday flow variations according to Ort et al.[23, 25]

Sampling. Twenty-four hour composite raw sewage samples were collected daily, over a one-week period (9–15th March 2011) from 21 STPs.[22] All samples were collected in silanized glass, polyethylene terephthalate (PET), or high-density polyethylene (HDPE) containers and extracted within 12 h of collection. If this was not possible, the samples were frozen immediately after collection at −20 °C until analysis to prevent degradation.[26] Deuterated labeled internal standards (IS) for quantification were added before extraction. For each day, the total sewage volume in the STP influent was recorded

2.2. Analysis of sewage drug biomarkers

Analytical techniques. Each group participating in the study performed the analysis of sewage samples following their in-house validated analytical method and was requested to report the techniques and the main quality criteria used. Sewage drug biomarkers were chosen among parent drugs and/or the main urinary metabolites of the most used illicit drugs: cocaine (COC) and its main metabolite benzoylecgonine (BE), amphetamine (AMP), methamphetamine (METH), 3,4-methylenedioxy-methamphetamine (MDMA or Ecstasy), and 11-*nor*-9-carboxy-delta9-tetrahydrocannabinol (THC–COOH), the main metabolite of cannabis. Several laboratories did not determine all compounds, as they did not have all of the necessary validated methods. All study participants used solid-phase extraction (SPE), with polymeric cartridges (**Table 1**). Different preconcentration factors were applied depending on the sensitivity of the instrumentation used. The majority of analyses were

performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using triple quadrupole (QqQ) analyzers (12 out of 14 laboratories), due to its robustness and excellent sensitivity and selectivity (**Table 1**). Two participants applied high-resolution mass spectrometry (HR)-MS, using time-of-flight or Orbitrap analyzers for quantification and confirmation.[32, 33] Both HR accurate mass analyzers have shown excellent performance for qualitative, and also for quantitative analysis of SDBs in sewage.[34]

Table 1. Different SPE, LC, and MS configurations used by the participants of this study for the analysis of sewage drug biomarkers (SDBs)[a]

configuration	SPE cartridges	LC separation	preconcentration factor	refs
LC-MS/MS QqQ	Oasis HLB (6 mL, 200 mg) (3)	YMC Hydrosphere C_{18} (1)	20−1500	7−9, 18, 27−30
SPE-offline (10)	Oasis HLB (3 mL, 60 mg) (1)	Acquity UPLC BEH C_{18} (2) and C_8 (1)		
	Oasis MCX (6 mL, 150 mg) (3)	Synergy POLAR-RP (1)[b]		
	Oasis MCX (3 mL, 60 mg) (3)	X-Bridge C_{18} (1)		
		X-Terra C_{18} (2)		
		X-Bridge Phenyl (1)		
		Phenomenex Luna HILIC (1)		
LC-MS/MS QqQ SPE-online (2)	Polymeric PLRP-s Hypersil C_{18} Gold PFP	Purospher Star RP-18 end-capped Hypersil C_{18} Gold Phenyl	(loading an aliquot of 5 mL)	31
LC-TOF MS (1)	Oasis MCX (6 mL, 150 mg)	Nucleosil C_{18}HD	200	32
LC-LTQ Orbitrap MS (1)	Oasis HLB (6 mL, 150 mg)	XBridge C_{18}	200	33

[a]Number of laboratories in parentheses. [b]This column was used for compounds analyzed under positive ion mode. For the determination of THC−COOH (in negative ion mode), the column used was Kinetex XB C_{18}.

Interlaboratory study. An interlaboratory study (ILS) was organized to provide information on the variability resulting from the analytical measurements by each of the participating laboratories. Two vials (ILS 1 and 2) containing known concentrations of the selected analytes in methanol were prepared by one group and sent blind to each participating laboratory. Each laboratory was asked to quantitatively determine analyte concentrations in ILS 1 and ILS 2 by analyzing three independent replicates from each solution, using their own in-house analytical method, and report the mean value of the triplicate results. Two data sets for the six analytes of interest were obtained. The number of laboratories (*n*) that submitted data were 12 for COC; 11 for AMP, METH, BE, MDMA; and 9 for THC–COOH. The ILS data submitted were tested for the normality of the distributions (Shapiro-Wilk test) and presence of outliers (Grubbs or Veglia), and z-scores were calculated from raw data. Z-scores are an internationally accepted measure for the performance of an individual laboratory with regard to the

group average.[35] The variability in the mean of laboratories provides an indication of how closely laboratory results match each other at a particular concentration. The results may disclose systematic errors or poor performance in individual laboratories, i.e., if a laboratory has more than two outlying values (in a similar direction) or z-scores outside a predefined level (usually >2.1). Details of the statistical analysis are available in a separate report.[36]

2.3. Stability of drug biomarkers in sewage

The stability of the selected SDBs was evaluated by a literature search in order to identify the degree of uncertainty that may arise from biotransformation in sewage. The evaluation considered both the behavior of the SDBs in the sewer system (from place of excretion to sample collection) and the stability of these compounds in the matrix during and after collection.

2.4. Back-calculation of drug use

The daily loads of each SDB in sewage were calculated by multiplying its concentration (ng/L) by the daily flow rate (m^3/d) measured during the sampling campaign as reported elsewhere.[22] Back-calculation of the amount of a drug used in a community is then achieved by taking into account, through proper correction factors, the stability of SDBs in sewage, human metabolism, and the dose used.[1, 13] The present study aims at improving this methodology by refining the correction factors, and by evaluating the uncertainty related to the back-calculation of use. Cocaine was chosen for this preliminary study because its excretion profile is relatively complete and available for the main routes of administration (i.e., intravenous (IV), intranasal (IN), oral (O), and smoked (SM)). Available data from pharmacokinetic studies regarding the excretion profile of the main COC metabolites were reviewed. The mean percentages of excretion of BE, the most abundant metabolite of COC, chosen for the back-calculation of consumption,[13] have been collected and analyzed. The mean percentage of excretion of BE was calculated for each route of administration and by weighting the mean excretion of each study by the number of subjects included (**Table S5**). The total uncertainty related to the back-calculation procedure has been evaluated for the first time as the relative standard deviation (RSD) of the mean BE

percentage of excretion. The present procedure was finally used to propose a refined correction factor for the back-calculation of COC use.

2.5. Estimation of population size

To facilitate the comparison of illicit drug estimates from sewage analysis among different cities, it is necessary to normalize the data to the number of inhabitants served by each corresponding STP. Information about census data and design capacity of the STPs were collected and hydrochemical parameters such as biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen (N), and phosphorus (P) were recorded for samples when possible. Specific loads for hydrochemical parameters (i.e., per-capita loads from domestic and, if available, industrial activity) were then used to calculate population equivalents for each sample following already established methodologies.[37] These values have been used together with census data to determine the most appropriate estimate for the population served by each individual STP.

3. Results and discussion

3.1. Sewage sampling and catchment characterics

Return rate of questionnaires. Nineteen of the 21 questionnaires sent to STPs to assess the suitability of sampling could be meaningfully evaluated; one was not returned, and one did not provide sufficient information to category I questions, i.e., only 7/15. On average, 13/15 category I questions (**Table S1**) were answered (category II, 10/13; category III, 7/10; category IV, 7/9) with large differences among STPs ranging from only a total of 3 to 21 missing answers. Detailed information about the evaluation of the questionnaires is reported in SI (Excel spreadsheet).

Sampling mode. In 11 STPs, a suitable sampling mode was specified: it weights individual discrete samples during the sampling period according to the momentary flow rate in the sewer to form a *nonbiased composite sample* (ten volume- and one flow-proportional).[23] The volume-proportional mode with sampling intervals varying according to flow rates in the sewer is conceptually not perfect, because individual sample volumes are constant.[23] However, in combination with short sampling intervals

(see later) it is acceptable, and most commercially available autosamplers are operated in this mode. In contrast, seven STPs indicated a time-proportional sampling mode that only results in unbiased composite samples either if flow variations are small or if concentration variations are not correlated with flow variations. It is difficult to assign a value for "small flow variations" and impossible to quantify the effect if the concentration profile over time within any day is unknown. High temporal resolution flow data and concentration profiles would be necessary for a proper assessment of sampling bias. Intraday concentration profiles are lacking for obvious reasons, and flow profiles at higher temporal resolution than 1 h were only provided by three STP (1, 5, and 11 min). Therefore, the max/min flow ratio for typical dry weather conditions was considered as an indicator of the intraday flow variation: in two STPs it was smaller than two, while for the other five STPs it ranged from 3.4 to 5.4 or could not be determined. For a hypothetical case with an intraday max/min flow ratio of 10,[24] systematic sampling errors on any individual day on the order of 5–20% (RSD, i.e., 15–60% as 95% CI) were simulated. To prevent systematic under- or overestimation, it is necessary to use a flow- or volume-proportional instead of a time-proportional sampling mode.

Sampling frequency. The sampling frequency required to minimize random errors mainly depends on the magnitude of short-term (i.e., minutes) variations in SDB concentrations, which in turn depend on the expected number and duration of sewage packets containing the substance of interest. This number is dependent on population size, the number of users and pharmacokinetics, and the operation of pump stations in the catchment. In this study, we estimated the expected sampling uncertainty for a substance that is used by 1% of the population. Typically, there are approximately five toilet flushes per person per day.[38] To avoid an underestimation of sampling uncertainty, we assume that significant amounts of SDBs are excreted and discharged only with two toilet flushes per drug user and day. With these conservative assumptions, the random sampling uncertainty in fourteen STPs is expected to be less than 5% on an individual day (RSD, or 10% as 95th percentile). This was calculated with the above assumptions on prevalence and number of relevant toilet flushes according to Ort et al.[25] Only a much lower prevalence than assumed in the above example would substantially increase the sampling uncertainty with the given sampling setups. In five STPs, sampling

errors may exceed this value due to either inadequate sampling intervals (up to two hours) or intermittently operated lift stations in the catchment resulting in large load variations. Best practice suggestions related to sampling frequency depend on a combination of drug prevalence and catchment characteristics and are outlined in the subsequent section.

Catchment characteristics. Relevant catchment characteristics are summarized in the SI. From our point of view, the following pieces of information are most important: (1) Exfiltration: it implies potential loss of SDBs and could be either considered directly in the back-calculation process or explicitly reported with the data for comparison with other cities. Neglecting this aspect typically would lead to an underestimation of illicit drug consumption. (2) Special events: holidays, parties, or discovery of illicit drug production facilities can lead to unusual SDB loads in sewers. It is important to report any known observations with the data, even if they are only speculative. (3) Layout of STPs and sewer catchments: any aspects that might affect the mass flux of SDBs need to be reported. These may be standard operation (e.g., routinely diverting part of the influent) or changes in the catchment (e.g., exceptional treatment of additional wastewater volumes from another part of the city).

The information collected with the questionnaires allows for an identification of the most appropriate sampling setups and critical cases. For large catchments and STPs as those investigated in this study, most of the current sampling protocols seem to be appropriate to keep random sampling uncertainty below 5–10% (RSD). This is consistent with previous findings.[20, 21] If such in-depth analyses cannot be performed, it is recommended to aim for a flow- or volume-proportional setup with sampling intervals not exceeding 10 min. Even shorter sampling intervals (down to 1 min) must be adopted in the following cases: (i) small(er) catchments or (ii) effluents of individual premises or buildings such as hospitals or prisons. In these cases, a flow-proportional sampling setup with precautionary short sampling intervals is a must to anticipate unknown or unusual (hydraulic) events. Finally, the meta-data should be carefully collected during each sampling campaign and stored with the analytical data, since they can be different for each STP and hence not generalizable. The questionnaire

provides guidance by listing the most common and important aspects and allows collection of any comments that seem relevant in a formalized manner.

3.2. Analysis of sewage drug biomarkers

Analytical quality criteria. The determination of sewage drug biomarkers has been performed following a specific analytical protocol to ensure acceptable analytical quality criteria (SI for details). Each of the 12 laboratories reported analyte concentrations as the mean of 3 measurements (replicate analysis). An important aspect to ensure the correct quantification in LC-MS/MS methods is the correction for matrix effects and for potential errors associated to sample manipulation and storage. In this study, with very few exceptions, each compound was corrected by the use of labeled IS, added to the samples before sample treatment (**Tables S2 and S3**). To have an indication of the method performance of each laboratory, the total analytical variability was calculated for each compound from the RSD of triplicate analyses of each sample. Overall results ranged from 1% to 34% (COC 1–13%; BE 1–14%; MDMA 3–22%; AMP 4–34%; METH 3–28%; THC–COOH 8–21%), which seems consistent with those calculated from the ILS (see below). One of the best options to evaluate the laboratory performance and interlaboratory variability would be to perform ILS on fortified sewage samples (e.g., in six replicates); this should be considered in subsequent investigations.

Efforts have been made to coordinate and harmonize the criteria for the estimation of realistic limits of detection (LODs) and limits of quantification (LOQs). Several factors such as preconcentration factor applied by each participant and matrix effects make their estimation problematic. In order to evaluate on the lowest concentrations that could be detected and satisfactorily quantified, participants were asked to report the lowest calibration levels employed (**Tables S2 and S3**).

For the confirmation of positives, we followed the guidelines of the European Decision 2002/657/EC,[39] which is based on the collection of identification points (IPs). The number of IPs earned depends on the mass analyzer used. A minimum of two transitions should be monitored for a reliable positive finding in tandem MS low mass resolution instruments. For HR instruments, at least two ions need to be monitored. Conformity of

the ion ratio between both recorded transitions and retention time with those of standards is required within the maximum tolerances allowed. Notable differences in the ion ratios were observed among the participants even when using the same transitions, surely due to the different instrument manufacturers and the instrumental conditions selected by each participant (**Tables S2 and S3**).

Interlaboratory variability. **Table 2** reports the results of the ILS. The mean of laboratory means, standard deviations, and the relative standard deviation (RSD) are presented for the six SDBs considered in the present study using raw data and data from which outliers have been removed. All data sets showed a normal distribution. The interlaboratory variability for the raw data ranges from 12% to 26% for ILS 2 and from 26% to 38% for ILS1. When outliers are removed, the overall variability ranges from 6% to 26% for ILS2 and 11% to 23% for ILS1; the highest values (26% and 23%) are referred only to methamphetamine analysis and the other values are below 18%.

Table 2. Results of the interlaboratory study – ILS (concentration in μg/L)

	Raw Data							
	ILS 1 Nominal value: 50 μg/L				ILS 2 Nominal value: 500 μg/L			
compounds investigated	average[a]	SD[b]	RSD[c] %	n^d	average[a]	SD[b]	RSD[c] %	n^d
cocaine	51.6	15.4	30	12	471	75.7	16	12
benzoylecgonine	54.9	15.5	28	11	511	61.3	12	11
amphetamine	54.1	14.2	26	11	513	96.8	19	11
methamphetamine	49.1	18.1	37	11	457	117.2	26	11
MDMA	55.3	16.6	30	11	507	93.6	18	11
THC−COOH	60.5	23.0	38	9	525	61.9	12	9
	Data after Removal of Outliers							
	average[a]	SD[b]	RSD[c] %	$n_G{}^e$	average[a]	SD[b]	RSD[c] %	$n_G{}^e$
cocaine	47.6	5.1	11	1	471	75.7	16	0
benzoylecgonine	50.6	6.2	12	1	487	30.6	6	2
amphetamine	50.6	8.7	17	1	473	43.9	9	2
methamphetamine	44.5	10.3	23	1	457	117.2	26	0
MDMA	48.3	5.7	12	2	507	93.6	18	0
THC−COOH	53.2	7.5	14	1	525	61.9	12	0

[a]Average of laboratory means. [b]SD, standard deviation. [c]RSD, relative standard deviation in percentage. [d]n, number of lab means used for calculation. [e]n_G, number of Grubbs outliers.

Laboratory performance. *Z*-scores are provided in **Table S4**. *Z*-scores, which can be negative (laboratory mean is below group average) or positive (lab mean above group average) provide an indication of the accuracy of each laboratory and the presence (or absence) of systematic errors. Laboratories with a consistent z-score ≥2, but with random negative and positive scores, have relatively poor accuracy. A

consistently negative or consistently positive z-score ≥2 is an indication of a systematic bias, and the results from a corresponding lab were excluded from further interpretation. Overall, z-scores were considered satisfactory (**Table S4**), with the exception of one laboratory that had consistently positive z-scores, the majority of which (8 out of 12) were ≥2 and could influence the overall variability. We could conclude that RSDs ranging between 6% and 26% can be expected from analytical detection only at the concentrations analyzed here (S1 0.06–0.5 ng and S2 0.6–5 ng absolutely injected), provided that laboratories have full control of their in-house analytical method.

The current study underlines that ILS are imperative for regular checks of quality control, including the assessment of accuracy and possible biases. The presented ILS data only consider direct analysis of SDBs in the absence of any matrix, while the total analytical variability in real samples was evaluated only on the triplicate analysis of each sample. It is realistic to expect that, within an ILS conducted on sewage samples, the uncertainty could increase, and this should be considered in future investigations.

3.3. Stability of drug biomarkers in sewage

Stability in the sewage system. **Table 3** gives an overview of the available laboratory experiments performed to assess the stability of SDBs in sewage. These data provide an indication of the extent of SDBs biotransformation that may occur during in-sewer transport. Amphetamine-like stimulants (AMP, METH, and MDMA) are generally stable (no loss of analyte in sewage) up to 72 h at 4 °C[27] and up to 12 h at 20 °C.[43] These observations are in line with stability studies for these three compounds in urine at 37 °C for 3 days and longer.[44] THC–COOH is also stable at the experimental conditions investigated, as well as BE, the main metabolite of COC. The observed changes are due to a partial degradation (hydrolysis) of COC to BE, also detected in blood and urine.[45] Instead, COC and its metabolite ecgonine methyl ester (EME) are less stable in sewage.[42] The variability observed between the different studies is probably due to the different conditions used in the experiments (temperature, pH, matrix, and spiking concentrations), and can be influenced deeply by the nature and composition of sewage.[42]

Table 3. Stability of Sewage Drug Biomarkers (SDBs) in sewage[a]

	references						
	Castiglioni et al. (2006)[27]	Gonzalez-Marino et al. (2010)[40]	Bisceglia (2010)[41]	Baker and Kasprzyk-Hordern (2011)[26]	Castiglioni et al. (2011)[42]	van Nuijs et al. (2012)[43]	unpublished results NIVA
	72 h 4 °C, pH 7.5	24 h 4 °C, pH 7.5	12 h 23 °C, pH 7.4	12 h 19 °C, pH 7.4	24 h 4 °C, pH 7.5	12 h 20 °C, pH 7.5	3.5 h 37 °C, pH 2
COC	−36%	−7%	−50%	−8%	−25%	−40%	0%
BE	+14%	+7%	+10%	+7%	+20%	+6%	0%
EME[b]	NA	NA	−40%	NA	−50%	−20%	NA
AMP	+5%	+0%	−15%	+47%	NA	+3%	NA
METH	+0%	NA	+0%	+8%	NA	+2%	NA
MDMA	+1%	NA	+0%	+1%	NA	+3%	NA
THC−COOH	−8%	+2%	NA	NA	NA	NA	NA

[a]+ and − are referred to the increase or decrease of SDBs in sewage at the end of the stability test. NA = not analyzed. [b]EME = Ecgonine methyl ester.

Considering the estimated mean residence time of sewage in the sewer systems investigated (0.5–15 h), the loss or formation of SDBs (except COC and EME which were not selected for back-calculation because of their instability in sewage) is generally lower than 10%, indicating therefore a negligible uncertainty. However, any potential increase of COC in-sewer biotransformation can produce higher amounts of BE and an increase of the uncertainty related to the back-calculation procedure.

Recently, it has been shown that two other phenomena which can occur in sewage, e.g., adsorption onto the solid particulate matter (SPM) and the conjugation of drug residues, are not important factors affecting the uncertainty. In particular, the percentage of adsorbed COC onto SPM was <3.1%, of BE <0.5%, of AMP <8.6%, of MDMA <2.4% and of METH <2.3%.[46, 47] Regarding the excreted metabolic conjugates, it is reported that they completely reverted to the parent compound in wastewater by bacterial activity.[27]

Stability during sampling, storage, and analysis. Another critical step for the stability of SDBs is the 24 h collection period that in most of the cases occurred at refrigerated conditions (4 °C). COC and EME are not stable under these conditions, and it is possible that the concentration of BE in the composite samples could increase by as much as 20% over 24 h at 4 °C (**Table 3**). By reducing the sample pH to 2, this could be prevented.[26] However, the concentration of BE in sewage is higher than that of COC (around double), so the relative contribution of hydrolyzed COC (after sample

collection) to the total BE concentration is small. The refrigerated conditions for sampling are sufficient for preventing transformation of the other investigated SDBs (**Table 3**).

The COC/BE ratio can be used to check for excessive biotransformation of COC to BE and for the presence of COC discharges other than human metabolism. According to human metabolism, the COC/BE ratio in wastewater should be around 0.1 or lower, but the ratios observed within this study were between 0.1 and 0.7 (**Figure S1**), in line with those found in previous studies.[42-48] This suggests a role for other sources of COC probably related to transport, handling, route of consumption, and other minor excretion routes (i.e., sweat) of the drug. In few cases (three STPs), refrigeration was not available during collection, and in these cases, the bias due to COC biotransformation could be higher.

Following collection, the samples were either frozen (−20 °C) or stabilized on SPE cartridges within 12 h, since according to published data, these conditions can prevent degradation of the selected SDBs.[26-34, 40-43]

In view of these results, the contribution of stability of SDBs to the total uncertainty of the procedure during in-sewer transport, sampling, storage, and analysis is expected to be minimal (<10%), if the proper procedures are adopted (see summary in **Table 5**).

3.4. Back-calculation of cocaine use

BE is the sewage biomarker commonly used for estimating COC use by wastewater analysis. Human urinary excretion of BE collected from the literature ranged from 6.5% (SM administration) to 55% (O administration) of an administered dose of COC (**Table S4**) depending on the route of administration, the habits of consumption, the amount of a dose, and individual metabolism. **Table 4** reports the mean percentage of BE excretion calculated for each route of administration from the available studies and weighted by the number of subjects involved in each study. No relevant differences were found after IV and IN administration, while SM administration gives a lower excretion possibly due to COC losses during smoking. Moreover, differences in BE

excretion were also observed among the subjects treated through the same route of administration, and the variability was higher for SM administration (RSD 40–60%) compared to IN and IV administration (RSD 24–27%). Another factor that can cause BE urinary excretion to decrease is the co-consumption of alcohol with COC.[49, 50]

Table 4. Mean percentages (%) of BE excretion for the different routes of administration (RoA) of COC[a]

	IN (9 studies, 56 subjects)	IV (7 studies, 28 subjects)	SM (3 studies, 20 subjects)	O[b] (1 study, 2 subjects)
means of all studies	32.1 ± 8.6 (26.9)	39.1 ± 9.3 (23.9)	16.3 ± 9.8 (59.8)	55 ± 7.1 (12.9)
means weighted by subjects	29.4 ± 7.4 (25.3)	37.3 ± 9.6 (25.8)	14.8 ± 5.8 (39.1)	-
mean of BE Excretion (IN, IV, and SM RoA)		27.1 ± 11.4 (41.9)		-
mean of BE excretion weighted by RoA[c]		29.2 ± 7.8 (26.5)		-

[a]Means ± standard deviation and relative standard deviation (RSD) in brackets. See Table S5 for raw data and references. IN = intranasal administration; IV = intravenous administration; SM = smoked administration; and O = oral administration. [b]This value was not further considered in the analyses because it referred only to 2 subjects and to a minor RoA. [c]Assuming the following pattern of consumption of COC: 95% used IN, 2% used IV, and 4% used SM.[51]

The high variability (RSD 42%) observed for BE excretion weighted by subjects (mean = 27%) is thus ascribable to the different patterns of BE excretion among the different routes of administration. The frequency of COC use for the different routes of administration might also be considered to weight BE excretion data, because it is generally far higher for IN administration than for IV and SM. Due to the generally scattered information referred strictly to "consumers under treatment", which represents just a minor group of COC users, only the results of a recent multicenter European study[51] could be used to figure out the pattern of COC use in the general population (**Table 4**). The obtained mean BE excretion was 29% of a COC dose with a variability (RSD 26%) lower than that calculated for data not weighted by the frequency of COC use (RSD 42%). Considering this excretion value, a novel refined correction factor of 3.59, calculated as previously described,[13] can be proposed to standardize the back-calculation of COC use. Nevertheless, this procedure requires fine-tuning to local data, since the preferential route of COC administration can be different from country to country.[51]

The uncertainty related to the back-calculation of drug use,[1, 2, 13] shown here for cocaine as an example, was calculated for the first time from a complete review of accessible literature and demonstrates how variable the excretion profile of a SDB can

be. This variability can influence the reliability of drug estimates obtained from sewage analysis; therefore, the use of proper correction factors is recommended in order to reduce uncertainty and improve the comparability of results (**Table 5**).

Table 5. "Best Practice" requirements for the different steps involved in the estimation of community drug use through the measurement of Sewage Drug Biomarkers (SDBs) and uncertainty values assessed under these conditions

steps considered	"best practice" requirements	uncertainty (%)
sampling	sampling mode: (a) flow-proportional; (b) volume proportional; (c) time-proportional (only for small flow variations)	5−10
	sampling frequency: depending on SDBs concentration variations (population size, number of users, pharmacokinetics, operation of pump stations)	
	catchment characteristics: exfiltration, special events, layout of STPs and sewer catchment affecting mass flux of SDBs	
chemical analysis of SDBs	analytical quality: use of labeled IS, use of internal quality controls, estimation of comparable LODs and LOQs, confirmation of positives	1−34 real samples analyses
	interlaboratory variability and laboratory performance: interlaboratory study (ILS) are mandatory on standards and real samples, assessment of accuracy and biases	6−26 from ILS
stability of SDBs	sewage system: resident time of wastewater in sewer before sampling	<10
	during sampling, storage, analysis: 24 h collection at refrigerated conditions, samples acidification (pH 2), freeze samples (−20 °C), or stabilize on SPE cartridges immediately after collection	
back-calculation of consumption	selection of proper SDBs (see refs 1, 2, 13.)	26 (cocaine case)
	collection of excretion profiles for all the routes of administration, weight for the number of subjects and the frequency of use	
estimation of population size	collection of all available data: estimates from hydrochemical parameters, census data	7−55
	choose the most adequate (combination of) value case by case with expert knowledge from experienced STP staff. Although widely available, design capacities are not recommended, as design rules in different countries vary and, furthermore, without knowing the planning horizon and actual loading (recent hydrochemical parameters), it is unknown whether the STP runs above or below design capacity.	

3.5. Estimation of population size

A comparison among the population estimates calculated from hydrochemical parameters and the other collected data (i.e., census data and design capacity) has been performed for each catchment investigated. **Table S6** summarizes the different population estimates collected for each catchment that were used to calculate the overall variability. It is generally high ranging between 7% and 55% (RSD) and depending on factors that could not easily be controlled such as the composition of sewage (i.e., industrial, domestic, or mixed) that can influence the hydrochemical parameters, the reliability of census data, the methods used to calculate population equivalents. The quality of the measured flow data (i.e., daily sewage volumes) used to estimate population equivalents can also influence variability, even if the systematic flow measurement errors cancel out when population size is determined from hydrochemical parameters (e.g., BOD).[20] Considering a mean value of all the collected

data to estimate the population size served by each plant is therefore not deemed appropriate due to the large biases that could be introduced into the final calculations of drug use estimates. The only feasible strategy to significantly reduce uncertainty was to determine the most reliable estimation of population size case by case using expert judgment from STP personnel. Experienced personnel know the contribution of specific industries toward the load of specific hydrochemical parameters and can select the one that is least influenced by industry reflecting better the number of people served by the STP. The estimation of population size in a STP catchment should be further improved by exploring the possibility to use suitable biomarkers consumed or excreted in known amounts by a population (i.e., prescription drugs, creatinine).[20, 52-54]

In conclusion, uncertainty could be assessed for the different steps involved in the estimation of community drug use through the measurement of SDBs. Provided that the proper procedures are employed, uncertainties (RSD) can be expected as follows: 5–10% for sampling; 6–26% from ILS, and 1–34% from replicated analysis of samples for chemical analysis; 10% for stability of SDBs; 26% for back-calculation of cocaine use. The highest biases are related to the analysis of SDBs and to the back-calculation of drug use, while for the estimation of population size, the only reasonable procedure is to choose the "most reliable value" case by case, due to the high variability observed among the estimates calculated from different approaches (7–55%). For each step, a "best practice" procedure has been suggested and discussed in order to reduce and/or keep minimal, where possible, the uncertainty of the entire procedure and improve the reliability of the estimates of drug use (**Table 5**). Currently, the most urgent needs for future research are as follows: (1) improve the quality of sewage chemical analyses by following specific quality requirements; (2) improve the knowledge on stability of SDBs in-sewer and during sampling to solve some of the critical issues raised before; (3) plan additional pharmacokinetic studies for the main classes of illicit drugs in order to produce reliable human excretion profiles for SDBs; (4) explore novel possibilities to estimate the population size in a catchment.

Supporting information

Additional information about the catchments under investigation, questionnaires elaboration results, analytical quality criteria and cocaine pharmacokinetic studies considered in the present study. A further improved version of the questionnaire is available from authors upon request. This material is available free of charge via the Internet at http://pubs.acs.org.

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References

1. van Nuijs, A. L. N.; Castiglioni, S.; Tarcomnicu, I.; Postigo, C.; Lopez de Alda, M. J..; Neels, H.; Zuccato, E.; Barcelo, D.; Covaci, A.Illicit drug consumption estimations derived from wastewater analysis: A critical review Sci. Total Environ. 2011, 409, 3564– 3577
2. Castiglioni, S.; Zuccato, E.; Fanelli, R., Eds. Illicit drugs in the environment. Occurrence, Analysis, and fate using mass spectrometry; John Wiley& Sons: Hoboken, NJ, 2011.
3. Daughton, C. G.Illicit Drugs in Municipal Sewage: Proposed New Non-Intrusive Tool to Heighten Public Awareness of Societal Use of Illicit/Abused Drugs and Their Potential for Ecological Consequences. In Pharmaceuticals and Personal Care Products in the Environment, Scientific and Regulatory Issue, Daughton, C. G.; Jones-Lepp, T. L., Eds.; ACS Symposium Series 791; American Chemical Society: Washington, DC, 2001; p 348.
4. Zuccato, E.; Chiabrando, C.; Castiglioni, S.; Calamari, D.; Bagnati, R.; Schiarea, S.; Fanelli, R.Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse Environ. Health 2005, 4, 14
5. Irvine, R. J.; Kostakis, C.; Felgate, P. D.; Jaehne, E. J.; Chen, C.; White, J. M.Population drug use in Australia: a wastewater analysis Forensic Sci. Int. 2011, 210, 69– 73
6. Metcalfe, C.; Tindale, K.; Li, H.; Rodayan, A.; Yargeau, V.Illicit drugs in Canadian municipal wastewater and estimates of community drug use Environ. Pollut. 2010, 158, 3179– 85
7. Terzic, S.; Senta, I.; Ahel, M.Illicit drugs in wastewater of the city of Zagreb (Croatia)-estimation of drug abuse in a transition country Environ. Pollut. 2010, 158, 2686– 2693
8. Karolak, S.; Nefau, T.; Bailly, E.; Solgadi, A.; Levi, Y.Estimation of illicit drugs consumption by wastewater analysis in Paris area (France) Forensic Sci. Int. 2010, 200, 153– 160

9. Reid, M. J.; Langford, K. H.; Mørland, J.; Thomas, K. V.Quantitative assessment of time dependent drug-use trends by the analysis of drugs and related metabolites in raw sewage Drug Alcohol Depend. 2011, 119, 179– 86
10. Berset, J. D.; Brenneisen, R.; Mathieu, C.Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-MS/MS) Chemosphere 2010, 8, 859– 866
11. Banta-Green, C. J.; Field, J. A.; Chiaia, A. C.; Sudakin, D. L.; Power, L.de Montigny, L. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxy-methamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal wastewater Addiction 2009, 104, 1874– 1880
12. van Nuijs, A. L. N.; Pecceu, B.; Theunis, L.; Dubois, N.; Charlier, C.; Jorens, P. G.; Bervoets, L.; Blust, R.; Meulemans, H.; Neels, H.; Covaci, A.Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium Addiction 2009, 104, 734– 41
13. Zuccato, E.; Chiabrando, C.; Castiglioni, S.; Bagnati, R.; Fanelli, R.Estimating community drug abuse by wastewater analysis Environ. Health Perspect. 2008, 116, 1027– 1032
14. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Assessing illicitdrugs in sewage - Potential and limitations ofa new monitoring approach. Insight 9; Office for Official Publications of the European Communities: Luxembourg, 2008; http://www.emcdda.europa.eu/publications/insights/wastewater.
15. Postigo, C.; Lopez de Alda, M. J.; Barceló, D.Evaluation of drugs of abuse use and trends in a prison through wastewater analysis Environ. Int. 2011, 37, 49– 55
16. Gerrity, D.; Trenholm, R. A.; Snyder, S. A.Temporal variability of pharmaceuticals and illicit drugs in wastewater and the effects of a major sporting event Water Res. 2011, 45, 5399– 5411
17. Zuccato, E.; Castiglioni, S.; Tettamanti, M.; Olandese, R.; Bagnati, R.; Melis, M.; Fanelli, R.Changes in illicit drugs consumption patterns in 2009 detected by wastewater analysis Drug Alcohol Depend. 2011, 118, 464– 69

18. Bijlsma, L.; Sancho, J. V.; Pitarch, E.; Ibáñez, M.; Hernández, F.Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater J. Chromatogr., A 2009, 1216, 3078– 3089
19. Reid, M. J.; Harman, C.; Grung, M.; Thomas, K. V.The current status of community drug testing via the analysis of drugs and drug metabolites in sewage Nor. Epidemiol. 2011, 21, 15– 23
20. Lai, F. Y.; Ort, C.; Gartner, C.; Carter, S.; Prichard, J.; Kirkbride, P.; Bruno, R.; Hall, W.; Eaglesham, G.; Mueller, J. F.Refining the estimation of illicit drug consumptions from wastewater analysis: co-analysis of prescription pharmaceuticals and uncertainty assessment Water Res. 2011, 45, 4437– 48
21. Mathieu, C.; Rieckermann, J.; Berset, J. D.; Schürch, S.; Brenneisen, R.Assessment of total uncertainty in cocaine and benzoylecgonine wastewater load measurements Water Res. 2011, 45, 6650– 6660
22. Thomas, K. V.; Bijlsma, L.; Castiglioni, S.; Covaci, A.; Emke, E.; Grabiç, R.; Hernández, F.; Karolak, S.; Kasprzyk-Hordern, B.; Lindberg, R.; Lopez de Alda, M. J.; Meierjohann, A.; Ort, C.; Pico, Y.; Quintana, J. B.; Reid, M.; Rieckermann, J.; Terzic, S.; van Nuijs, A. L. N.; de Voogt, P.Comparing illicit drug use in 19 European cities through sewage analysis Sci. Total Environ. 2012, 432, 432– 439
23. Ort, C.; Lawrence, M. G.; Rieckermann, J.; Joss, A.Sampling for PPCPs and illicit drugs in wastewater systems: Are your conclusions valid? A critical review Environ. Sci. Technol. 2010, 44, 6024– 6035
24. Ort, C.; Schaffner, C.; Giger, W.; Gujer, W.Modeling stochastic load variations in sewer systems Water Sci. Technol. 2005, 52, 113– 122
25. Ort, C.; Lawrence, M. G.; Reungoat, J.; Mueller, J. F.Sampling for PPCPs in wastewater systems: A comparison of different sampling modes and optimization strategies Environ. Sci. Technol. 2010, 44, 6289– 6296
26. Baker, D. R.; Kasprzyk-Hordern, B.Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid

phase extraction and liquid chromatography-mass spectrometry J. Chromatogr., A 2011, 1218, 8036– 8059

27. Castiglioni, S.; Zuccato, E.; Crisci, E.; Chiabrando, C.; Fanelli, R.; Bagnati, R.Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography – tandem mass spectrometry Anal. Chem. 2006, 78, 8421– 8429
28. Van Nuijs, A. L. N.; Tarcomnicu, I.; Bervoets, L.; Blust, R.; Jorens, P. G.; Neels, H.; Covaci, A.Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry Anal. Bioanal. Chem. 2009, 395, 819– 828
29. Kasprzyk-Hordern, B.; Dinsdale, R. M.; Guwy, A. J.Multiresidue methods for the analysis of pharmaceuticals, personal care products and illicit drugs in surface water and wastewater by solid-phase extraction and ultra performance liquid chromatography-electrospray tandem mass spectrometry Anal. Bioanal. Chem. 2008, 391, 1293– 1308
30. Vazquez-Roig, P.; Andreu, V.; Blasco, C.; Picó, Y.SPE and LC-MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (València, Spain) Anal. Bioanal. Chem. 2010, 397, 2851– 2864
31. Postigo, C.; Lopez de Alda, M. J.; Barceló, D.Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography-electrospray-tandem mass spectrometry Anal. Chem. 2008, 80, 3123– 3134
32. González-Mariño, I.; Quintana, J. B.; Rodríguez, I.; González-Díez, M.; Cela, R.Screening and Selective Quantification of Illicit Drugs in Wastewater by Mixed-Mode Solid-Phase Extraction and Quadrupole-Time-of-Flight Liquid Chromatography-Mass Spectrometry Anal. Chem. 2012, 84, 1708– 1717
33. Hogenboom, A. C.; van Leerdam, J. A.; de Voogt, P.Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography - hybrid linear ion trap Orbitrap mass spectrometry J. Chromatogr., A 2009, 1216, 510– 519
34. Bijlsma, L.; Emke, E.; Hernández, F.; de Voogt, P.Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography

coupled to high resolution mass spectrometry Chemosphere 2012, 89, 1399–1406

35. United Nation Industrial Development Organization. Complyingwith ISO 17025. A practical guidebook for meeting the requirementsof laboratory accreditation schemes based on ISO 17025: 2005 or equivalen*t national* standards: Vienna, 2006; http://www.labnetwork.org/en/testing-areas/chemical-lab/108-proficiency-testing#6
36. Asgadaouan, A. VIO 2011-DOA-EU-cities; Interlaboratory study; KWR: Nieuwegein, 2011.
37. Andreottola, G.; Bonomo, L.; Poggiali, L.; Zaffaroni, C.A Methodology for the Estimation of Unit Nutrient and Organic Loads from Domestic and Non-Domestic Sources Eur. Water Pollut. Control 1994, 4, 13– 19
38. Rauch, W.; Brockmann, D.; Peters, I.; Larsen, T. A.; Gujer, W.Combining urine separation with waste design: an analysis using a stochastic model for urine production Water Res. 2003, 37 (3) 681– 689
39. Commission Decision2002/657/EC of 12 August implementing Council Directive 96/23/EC concerning performance of analytical methods and the interpretation of results Off. J. Eur. Commun. 2002, L221/8
40. Gonzalez-Marino, I.; Quintana, J. B.; Rodriguez, I.; Cela, R.Determination of drugs of abuse in water by solid-phase extraction, derivatisation and gas chromatography-ion trap-tandem mass spectrometry J. Chromatogr., A 2010, 1217, 1748– 1760
41. Bisceglia, K. J. Ph.D. dissertation; Johns Hopkins University, Baltimore, 2010.
42. Castiglioni, S.; Bagnati, R.; Melis, M.; Panawennage, D.; Chiarelli, P.; Fanelli, R.; Zuccato, E.Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine Water Res. 2011, 45, 5141– 5150
43. van Nuijs, A. L. N.; Abdellati, K.; Bervoets, L.; Blust, R.; Jorens, P. G.; Neels, H.; Covaci, A.The stability of illicit drugs and metabolites in wastewater, an important issue for sewage epidemiology J. Hazard. Mater. 2012, 239–240, 19– 23

44. Jimenez, R.; de la Torre, C.; Ventura, M.; Segura, J.; Ventura, R.Stability studies of amphetamine and ephedrine derivatives in urine J. Chromatogr., B 2006, 843, 84– 93
45. Johansen, S. S.; Bhatia, H. M.Quantitative analysis of cocaine and its metabolites in whole blood and urine by high-performance liquid chromatography coupled with tandem mass spectrometry J. Chromatogr., B 2007, 852, 338– 344
46. Baker, D.; Kasprzyk-Hordern, B.Multi-residue determination of the sorption of illicit drugs and pharmaceuticals to wastewater suspended particulate matter using pressurised liquid extraction, solid phase extraction and liquid chromatography coupled with tandem mass spectrometry J. Chromatogr., A 2011, 1218, 7901– 7913
47. Baker, D.; Očenášková, V.; Kvicalova, M.; Kasprzyk-Hordern, B.Drugs of abuse in wastewater and suspended particulate matter — Further developments in sewage epidemiology Environ. Int. 2012, 48, 28– 38
48. van Nuijs, A. L. N.; Pecceu, B.; Theunis, L.; Dubois, N.; Charlier, C.; Jorens, P. G.; Bervoets, L.; Blust, R.; Neels, H.; Covaci, A.Spatial and temporal variations in the occurrence of cocaine and benzoylecgonine in waste- and surface water from Belgium and removal during wastewater treatment Water Res. 2009, 43, 1341– 1349
49. Harris, D. S.; Everhart, E. T.; Mendelson, J.; Jones, R. T.The pharmacology of cocaethylene in humans following cocaine and ethanol administration Drug Alcohol Depend. 2003, 72, 169– 82
50. Cami, J.; Farré, M.; González, M. L.; Segura, J.; de la Torre, R.Cocaine metabolism in humans after use of alcohol. Clinical and research implications Recent Dev. Alcohol 1998, 14, 437– 455
51. Prinzleve, M.; Haasen, C.; Zurhold, H.Cocaine use in Europe - a multi-centre study: patterns of use in different groups Eur. Addict. Res. 2004, 10, 147– 155
52. Harman, C.; Reid, M.; Thomas, K. V.Long-term measurement of population drug use – An approach using passive samplers in wastewater Environ. Sci. Technol. 2011, 45, 5676– 5682
53. Daughton, C. G.Real-time estimation of small-area populations with human biomarkers in sewage Sci. Total Environ. 2012, 414, 6– 21

54. Brewer, A. J.; Ort, C.; Banta-Green, C. J.; Berset, J.-D.; Field, J. A.Normalized diurnal and between-day trends in illicit and legal drug loads that account for changes in population Environ. Sci. Technol. 2012, 46, 8305– 8314

Chapter 6.3.3, scientific article c

Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis

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Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis

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Abstract

Aims To perform wastewater analyses to assess spatial differences and temporal changes of illicit drug use in a large European population. **Design** Analyses of raw wastewater over a 1-week period in 2012 and 2013. **Setting and Participants** Catchment areas of wastewater treatment plants (WWTPs) across Europe, as follows: 2012: 25 WWTPs in 11 countries (23 cities, total population 11.50 million); 2013: 47 WWTPs in 21 countries (42 cities, total population 24.74 million). **Measurements** Excretion products of five illicit drugs (cocaine, amphetamine, ecstasy, methamphetamine, cannabis) were quantified in wastewater samples using methods based on liquid chromatography coupled to mass spectrometry. **Findings** Spatial differences were assessed and confirmed to vary greatly across European metropolitan areas. In general, results were in agreement with traditional surveillance data, where available. While temporal changes were substantial in individual cities and years (*P* ranging from insignificant to $<10^{-3}$), overall means were relatively stable. The overall mean of methamphetamine was an exception (apparent decline in 2012), as it was influenced mainly by four cities. **Conclusions** Wastewater analysis performed across Europe provides complementary evidence on illicit drug consumption and generally concurs with traditional surveillance data. Wastewater analysis can measure total illicit drug use more quickly and regularly than is the current norm for national surveys, and creates estimates where such data does not exist.

Keywords

Amphetamine, cannabis, cocaine, drugs of abuse, ecstacy, methamphetamine, sewage.

Introduction

Illicit drug use is a covert and hidden activity that presents methodological challenges for drug surveillance systems. Questionnaire-based survey methods have traditionally been an important component of the approaches employed to monitor drug use, but it is recognized that these methods are not sufficient to monitor trends in drug use adequately and quickly, and require complementary data from other sources [1, 2]. The analysis of the excretion products of illicit drugs in wastewater [wastewater analysis (WWA)] has been explored since 2008 as an additional approach for estimating illicit drug use within specified regions, i.e. the catchment areas of wastewater treatment plants (WWTP) [3, 4]. While the approach cannot provide information on the behaviour of single users and on their demographics, there are a number of ways in which WWA can complement other survey methods and provide additional information to understand the illicit drug situation more clearly. Wastewater data can be obtained within short time-frames, are not prone to response biases and can help in identifying the spectrum of illicit drugs being used by a population. This is potentially important, given the emergence of new psychoactive substances [5]. Drug users are often unaware of the actual substance or mix of substances they are consuming, which makes self-report data unreliable. Wastewater analysis is therefore a potential approach to detect and estimate the use of new psychoactive substances; however, it should be noted that more information is necessary regarding their biotransformation pathways.

Wastewater analysis can provide information on daily, weekly, monthly and annual variations in illicit drug use. The weekly profile of cocaine and amphetamine-like stimulants use has already been assessed by collecting consecutive daily wastewater samples, which revealed higher use of these substances during weekends [6 - 12]. The monitoring of temporal trends in illicit drug consumption over a longer period of time (months) by WWA has been evaluated in three studies, and the major conclusions were that there was typically an increase of illicit drug use during holiday periods [11, 13, 14]. Wastewater analysis was further applied to detect yearly trends in illicit drug

consumption in Italy and Australia [15, 16]. In conclusion, this approach can provide important and timely information on short- and long-term trends in illicit drug use.

Wastewater studies in different countries have also detected regional variations in illicit drug use [17 - 22]. The influence of urbanization on the use of illicit drugs was evaluated in Oregon (USA) and South Australia and Queensland (Australia), concluding that the use of illicit drugs was higher in urban regions compared to more rural areas [9, 14, 23]. Wastewater analysis has also been applied to detect transnational differences in illicit drug use. The consumption of five substances was evaluated by analysing wastewater from 19 European cities for a 1-week period in 2011 [24]. Wastewater analysis can thus complement survey methods for a clearer understanding of actual spatial differences and temporal changes in illicit drug use.

However, until now no international study has been performed covering multiple countries over multiple years with a common protocol and adequate quality control measures. Therefore, the aims of this study were to:

1. collect wastewater samples from multiple European locations in 2012 and 2013;
2. calculate population-normalized mass loads of benzoylecgonine [BE; as indicator for cocaine (COC) use], amphetamine (AMP), methamphetamine (METH), ecstasy [3,4-methylenedioxy-methamphetamine (MDMA)] and 11-nor-9-carboxy-delta9-tetrahydrocannabinol [THC-COOH; as indicator for tetrahydrocannabinol (THC) use]; and
3. perform analytical quality control through inter-laboratory tests.

Methods

Sewer system characterization

Relevant information for each WWTP catchment was gathered systematically by means of a standardized questionnaire. An extended version of the questionnaire developed for earlier studies [24, 25] was used (Supporting information, Appendix S1). It comprises more than 50 questions classified according to importance. The number of the most important questions per category is indicated in brackets (year 2012/year

2013): General information (1/1), Catchment and population (2/5), Sewer system (2/2), WWTP influent (1/1), Sampling (5/5), Flow meter (3/3), Sample handling (9/9), Monitoring period (5/5).

Sampling and analysis

A 1-week period was targeted in 2012 (17–23 April) and 2013 (6–12 March). Daily 24-hour composite raw wastewater samples were collected over 7 consecutive days. Considering stability, metabolism and unambiguous indication of drugs actually having been consumed, the most suitable target residues were targeted: BE, AMP, METH, MDMA and THC-COOH [4]. It should be noted that the consumption of COC and THC was monitored through the analysis of their main metabolite because of higher concentrations and higher stability in wastewater.

Samples were spiked with isotope-labelled internal standards, either filtered and extracted immediately on solid-phase extraction cartridges or frozen at −20 °C until analysis. Each laboratory used fully validated analytical methods: target compounds present in the liquid phase of the wastewater were quantified in final extracts or with direct injection applying liquid chromatography coupled to tandem mass spectrometry or high-resolution mass spectrometry [25].

For quality assurance, each laboratory participated in yearly inter-laboratory tests (de Voogt *et al.*, unpublished). External quality control samples were evaluated (one standard in methanol and two fortified raw wastewater samples). A reliable estimation of the method limit of quantification (LOQ) was performed by evaluating the signal-to-noise ratio in these samples. In 2012, one of 14 laboratories did not meet the requirements for any compound in the inter-laboratory test and was excluded. In 2013, only METH results of one of 15 laboratories had to be excluded.

Calculations

Daily mass loads (g/day) of drug residues entering the WWTPs were calculated by multiplying measured concentrations (ng/L) in daily samples with the corresponding wastewater volumes (L/day). To compare cities of different sizes, mass loads are

normalized by the population size of the catchment (mg/1000 people/day). The estimated consumption of COC (section Benzoylecgonine) was back-calculated from the population-normalized mass loads of BE using a correction factor of 3.59 that takes the urinary excretion rate of COC into account for different dosages and routes of administration [25].

Uncertainty assessment

Mainly four components of uncertainty may affect the estimation of population-normalized drug loads: sampling (U_S), chemical analysis (U_C), flow rate measurement (U_F) and population estimation (U_P). Because the focus of this study is on mass loads in wastewater, uncertainties related to excretion rates and biodegradation in sewers are not considered. When estimating the overall uncertainty U_T of a mean value over an *n*-day monitoring period, uncertainty components that are random and independent on every day will be reduced by sqrt(*n*). This applies to U_S, as each sample is collected physically independent of the day before. All other components cannot be reduced by sqrt(*n*): (i) population is only estimated once, (ii) chemical analysis is carried out for all samples in one batch, and (iii) if a flow meter measures flows systematically incorrectly, it will be in the same direction every day. All components can be considered as independent. As long as U_S, U_C and $U_F \leq 30\%$ and $U_P \leq 10\%$ [relative standard deviation (RSD)], an estimation of U_T is valid with an approximative formula (e.g. [26]). A Monte Carlo simulation was used to avoid underestimating U_T systematically because a conservative estimate of U_P in our study is 20% (see Supporting information, Appendix S2).

Results

Table 1 lists participating cities: in 2012, 25 WWTPs in 11 countries were included (23 cities, total population 11.50 million); in 2013, there were 47 WWTPs in 21 countries (42 cities, total population 24.74 million). For comparison, 2011 data [24] were also used (21 WWTPs in 11 countries; 19 cities, total population 14.12 million). **Figures 1 - 5** summarize all results. Countries are ordered based on average loads over all years. The numbers in brackets indicate cities' overall ranks. While absolute variability within 1-week periods (grey range) is obviously higher for high loads, relative variability is not substantially

different throughout the entire load range and may vary from year to year, even within a location. The colour of the lines between the means indicate whether the change from 1 week in 1 year to 1 week in another year is significant (Wilcox, $\alpha = 0.05$). **Table 2** summarizes overall means, separately for cities that participated in all 3 years (cities in bold type in **Figs 1 - 5**) and for all cities per year (excluding cities that exhibited explainable anomalies, i.e. cities in italic type in **Figs 1 - 5**). Concentration values that were <LOQ were treated as follows: (1) if all values at a location for a certain compound were <LOQ, loads were set to zero; (2) if at least one value was >LOQ, values <LOQ were replaced with $0.5 \times$ LOQ. Dashed grey lines indicate a population-weighted overall mean for 2013 (all cities except cities in italics). When weekly patterns were evaluated in 2012, previous findings were confirmed, i.e. higher loads on weekends for BE, and MDMA and no substantial variation for AMP, METH and THC-COOH [24] (see Supporting information, Appendix S4).

Table 1. Summary of participating cities and wastewater treatment plants (WWTP). More detailed information can be found in Supporting information (Appendix S3), which includes raw data and answers from the questionnaire

Country	City WWTP	Population of the city under investigation ①	Estimated population in WWTP catchment						Targeted 1-week monitoring period ✓ (n=7days)			Loss of wastewater ②	Commuters ③	Special events ④
			2011	① method (year)	2012	① method (year)	2013	① method (year)	2011 Mar 9-15	2012 Apr 17-23	2013 Mar 6-12			
BA	**Sarajevo** Butile	C: 291422, M: 515012 (w)					130000	b (2013)		✓	✓	◔	▭	R (2013)
	Antwerp D. Deurne	498473 (e 2011)			213876	a (2011)	213876	a (2012)		✓	✓	◇	▭	
	Antwerp Z. Zuid	498473 (e 2011)	117200	a (2010)	130218	a (2011)	130218	a (2012)	✓	✓	✓	◇	▭	
BE	**Brussels** Noord	1136778 (e 2011)	1027300	b (2011)	953987	b (2012)	953987	b (2013)	✓	✓	✓	◇	▭	
	Geraardsbergen Geraardsbergen	32629 (w)					29047	c (2011)		✓	Mar 7-13	◇	▭	
	Koksijde Wulpen	31207 (w)					78441	a (2012)			Mar 21-27	◇	▰	
	Ninove Ninove	37295 (w)			260000	c (2012)	36179	c (2013)			✓	◇	▭	
	Basel ProRheno	C: 195743 (c 2013)			206655	c (2012)	260000	c (2012)		✓	✓	◔	▰	R (2012)
	Berne region bern	C: 137818 (c 2012)			410486	c (2012)	206700	c (2012)			Apr 21-27	◇	▰	R (2012)
CH	**Geneva** SIG	C: 194458 (c 2013)			89000	c (2012)	410500	c (2012)		Apr 22-28	✓	◇	▰	R (2012)
	St. Gallen Au and Hofen (2 WWTPs)	C: 74070 (c 2013)			410000	c (2012)	89000	c (2012)		✓ (n=5)	✓	◇	▰	R (2012)
	Zurich Werdhölzli	C: 394012 (c 2012)					410000	c (2012)		✓	✓	◇	▰	Y (2012)
	Nicosia Pano Deftera	234200 (e 2009)					28000	c (2012)			Mar 21-27	◇	▰	Y (2013)
CY	**Limassol** Amathus	185100 (e 2009)					272000	c (2010)			Mar 21-27	◇	▰	Y (2013)
	Budweis COV	93620 (e 2011)	112000	d (2010)	112000	d (2010)	110300	d (2013)	✓	✓	✓	◔	▰	Y (2013)
CZ	**Prague** UCOV	1241664 (e 2011)			1300000	c (2011)	1300000	c (2011)		✓	✓	◔	▭	
	Dortmund Deusen	580956 (e 2012)					371788	c (2010)			Mar 13-19	◇	▭	
	Dülmen Dülmen	46071 (w)					34495	c (2010)			✓	◇	▭	
DE	**Dresden** Kaditz	529781 (e 2012)					593050	c (2012)			✓	◇	▰	
	Copenhagen Lynetten	501285 (e 2003)					531000	c (2009)			Mar 6-13 (n=7)	◇	▭	
DK	**Barcelona** Baix Llobregat	C: 1620943; M: 3202571 (e 2012)	1162000	c (2007)	1162000	c (2010)	1162000	c (2010)	Mar 16-22	✓	✓	◇	▰	Y (2012/13) R
	Castellon Castellon de la Plana	C: 180204 (e 2012)	170600	a (2010)	204878	b (2012)	204878	b (2013)	✓	✓	Feb 20-26	◇	▭	
ES	**Santiago** Silvouta	95671 (e 2012)	136500	d (2010)	136500	d (2010)	136500	d (2010)	✓ (n=6)	✓	✓	◇	▰	
	Valencia Pinedo I+II* and QB** (3 WWTPs)	C: 797028 (e2012), M: 1353250 (e 2013)	1839000	a**/b* (2011)			1357952	e** (2011)/ b* (2013)	✓		✓	◇	▰	R (2011/13)

Continue next page

Country	City WWTP	Population of the city under investigation ⓪	Estimated population in WWTP catchment						Targeted 1-week monitoring period ✓ (n=7days)			Loss of wastewater ②	Com-muters ③	Special events ④
			2011	① method (year)	2012	① method (year)	2013	① method (year)	2011 Mar 9-15	2012 Apr 17-23	2013 Mar 6-12			
FI	**Helsinki** Viikinmäki	M: 1022139 (E 2009)	780000	a (2009)	780000	a (2009)	780000	a (2009)	✓	✓	✓	💧	-	
	Turku Kakola	178630 (E 2012)	*275000	d (2011)	275000	d (2011)	275000	d (2011)	✓ (n=6)	✓	✓	💧	-	R (2012)
FR	**Paris** Seine Centre[+]/Grésillon[++]	C: 2243718 (E 2010), M: 6507783 (E 2006)	[+]774600	b (2011)	[++]245500	f (2012)	[+]1004000	b (2013)	✓	Apr 21-27	✓	💧	🏷	Y (2012)
GB	**London** Beckton	8174000 (L 2011)	3400000	a (2010)			3400000	e (2010)	✓ (n=6)		✓	-	-	
GR	**Athens** Psyttalia	M: 2989023 (E 2009)					3700000	c (2011)			✓	💧	🏷	
HR	**Zagreb** Central	C: 688163, M: 1107623 (W)	650000	c (2001)	650000	c (2001)	650000	c (2011)	✓	✓	✓	-	🏷	
IT	**Milan** Nosedo	1295705 (E 2009)	1250000	c (2010)	1100000	b (2012)	1149477	b (2013)	✓	✓	✓	💧	🏷	
NL	**Amsterdam** West	779808 (E 2011)	694800	b (2011)	769000	c (2010)	769000	c (2010)	✓	Apr 17-25	✓	💧	🏷	Y (2012)
	Eindhoven Eindhoven	216036 (E 2011)	448700	b (2011)	450300	c (2005)	450300	c (2005)	✓	✓	✓	-	🏷	Y (2011/12)
	Utrecht Utrecht	311367 (E 2011)	297000	b (2011)	300000	c (2010)	300000	c (2010)	✓ (n=5)	✓	✓	💧	-	Y (2011)
NO	**Oslo** VEAS	599230 (E 2011)	557000	c (2009)	557000	c (2009)	576000	c (2012)	✓	✓ (n=6)	✓	💧	🏷	
PT	**Lisbon** Alcantara	C: 537412, M: 1860256 (E 2012)					426964	c (2011)			✓ (n=6)	💧	🏷	
RO	**Cluj Napoca** Cluj Napoca	304802 (E 2011)					350000	c (2011)			✓	-	-	
RS	**Belgrade** Sewer outlet Danube	C: 1232731, M: 1659440 (W)					284347	c (2008)			Mar 17-23 (n=6)	💧	🏷	
	Novi Sad Sewer outlet Danube	M: 341625 (W)					321282	d (2013)			Mar 14-19 (n=6)	-	-	R (2013)
SE	**Gothenburg** Ryaverket	513751 (E 2010)			664441	c (2011)	664441	c (2011)		✓	✓	💧	🏷	
	Stockholm Henriksdals (only 1 of 2 inlets)	C: 847073, M: 1550208 (E 2010)	315000	c (2009)					✓			-	-	
	Umeå Öns	115473 (E 2010)	115800	c (2010)	115800	c (2010)	115800	c (2010)	✓	✓	✓	💧	🏷	
SK	**Bratislava** Central and Petržalka (2	415589 (L 2012)					440000	c (2011)			✓	💧	🏷	Y (2013)
	Piestany Piestany	29660 (W)					30000	c (2011)			✓	💧	🏷	Y (2013)

⓪ Population of entire city/region C: City M: Metropolitan, greater region (E, W, L): Eurostat, Wikipedia and local bureau for population statistics (year) ① Method for population estimation in WWTP catchment (year of estimate); a, influent nutrient load over corresponding calendar year; b, influent nutrient load over actual sampling period; c, census d house connections / drinking water subscribers; e, values adopted from previous estimation; f, WWTP different from 2011/13 but wastewater from same catchment (central collection with subsequent distribution to different WWTPs). *Population estimate indicated in [24] was erroneous and population-normalized consumption estimates are corrected with updated value ② Loss of wastewater (exfiltration, questionnaire 2013)**.** , no loss expected; , loss indicated (unknown amount or <20%)**;** , loss >20% expected**;** -, information missing ③ Commuters (work days vs. weekend, questionnaire 2013). , no substantial net population in-/decrease due to commuters; net increase of population on **work days; -** information missing ④ Special events during/adjacent to monitoring period. Y, please see Supporting information Appendix S3 for type of event (year provided in brackets); R, rain before/during monitoring period (higher flows but no substantial effect on drug loads expected).

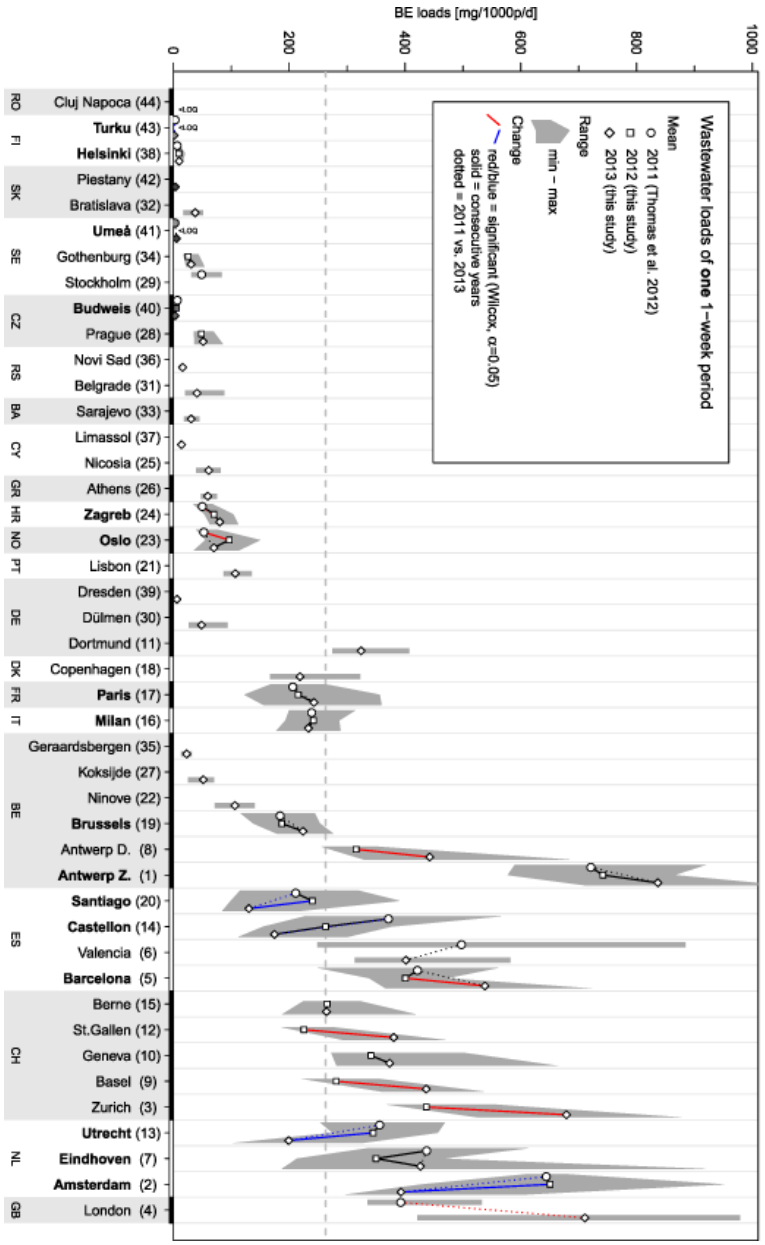

Figure 1. Population-normalized benzoylecgonine (BE) loads of a single 1-week period per year. See Table 1 for more information. **<LOQ**: concentrations in all daily samples were below limit of quantification (LOQ). **Grey dashed line**: 2013 overall mean of all participating cities. **Dot colour**: white: concentrations in all samples were above LOQ; grey shading: one or more concentrations were below LOQ and set to 0.5*LOQ (the darker the grey, the more concentrations were below LOQ). **Numbers in brackets**: cities' rank (average over all available years). Cities in **bold type** participated in all 3 years and were used to calculate annual overall means (see Table 2). All *P*-values can be found in Supporting information, Appendix S3

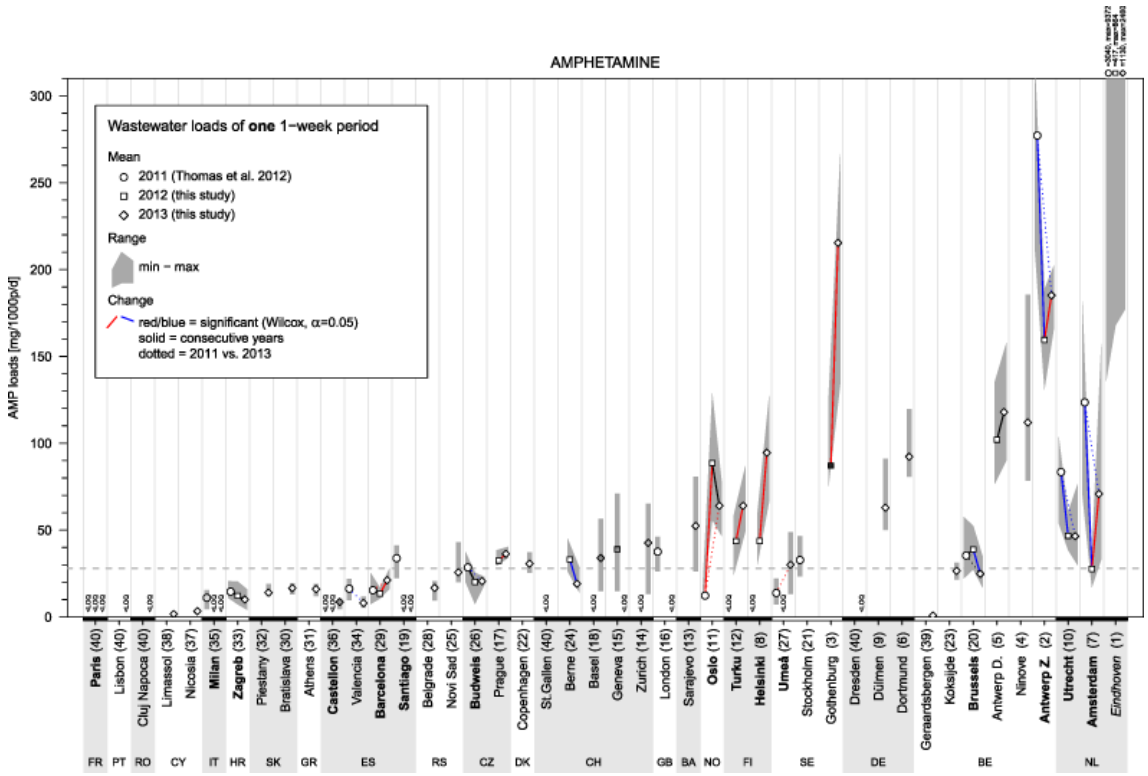

Figure 2. Population-normalized amphetamine (AMP) loads of a single 1-week period per year. See Table 1 for more information. **<LOQ**: concentrations in all daily samples were below limit of quantification (LOQ). **Grey dashed line**: 2013 overall mean of all participating cities (except Eindhoven). **Dot colour**: white: concentrations in all samples were above LOQ; grey shading: one or more concentrations were below LOQ and set to 0.5*LOQ (the darker the grey, the more concentrations were below LOQ). **Numbers in brackets**: cities' rank (average over all available years). Cities in **bold type** participated in all 3 years and were used to calculate annual overall means (see Table 2). Cities in **italic type** exhibited abnormal high values in at least 1 year (see text for more details). All *P*-values can be found in Supporting information, Appendix S3

Figure 3. Population-normalized methamphetamine (METH) loads of a single 1-week period per year. See Table 1 for more information. **<LOQ**: concentrations in all daily samples were below limit of quantification (LOQ). **Grey dashed line**: 2013 overall mean of all participating cities. **Dot colour**: white: concentrations in all samples were above LOQ; grey shading: one or more concentrations were below LOQ and set to 0.5*LOQ (the darker the grey, the more concentrations were below LOQ). **Numbers in brackets**: cities' rank (average over all available years). Cities in **bold type** participated in all 3 years and were used to calculate annual overall means (see Table 2). All *P*-values can be found in Supporting information, Appendix S3

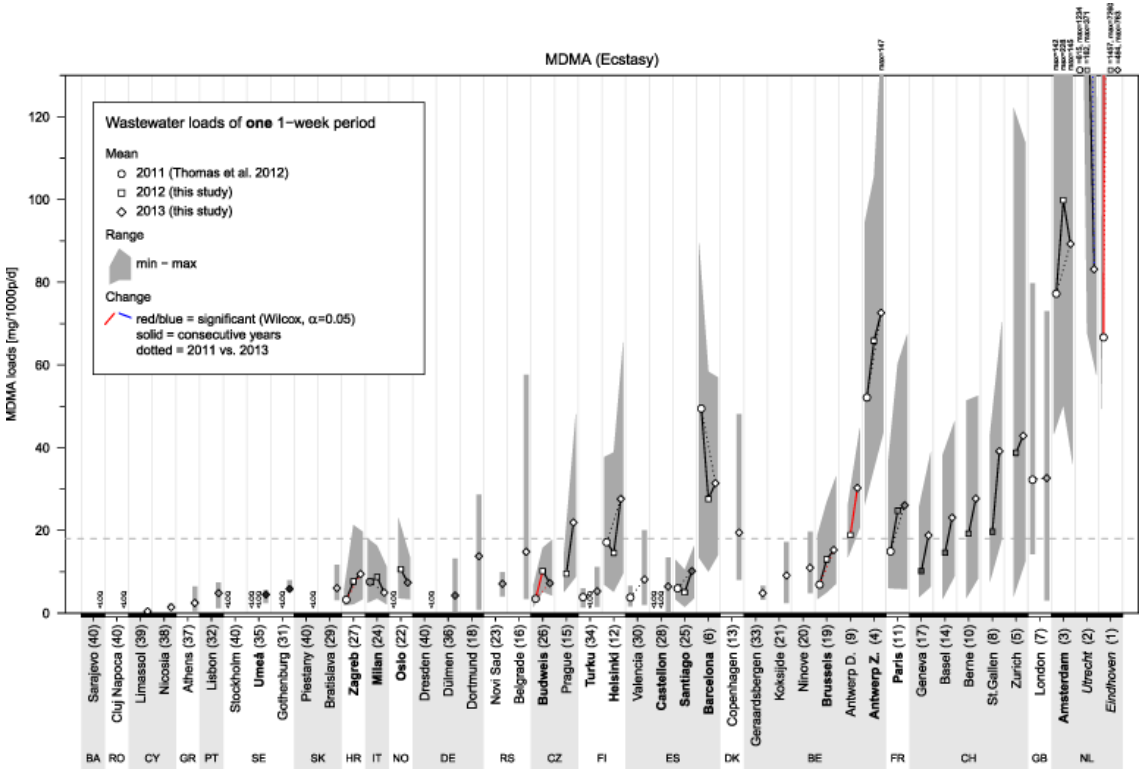

Figure 4. Population-normalized 3,4-methylenedioxy-methamphetamine (MDMA) loads of a single 1-week period per year. See Table 1 for more information. **<LOQ**: concentrations in all daily samples were below limit of quantification (LOQ). **Grey dashed line**: 2013 overall mean of all participating cities (except Utrecht and Eindhoven). **Dot colour**: white: concentrations in all samples were above LOQ; grey shading: one or more concentrations were below LOQ and set to 0.5*LOQ (the darker the grey, the more concentrations were below LOQ). **Numbers in brackets**: cities' rank (average over all available years). Cities in **bold type** participated in all 3 years and were used to calculate annual overall means (see Table 2). Cities in **italic type** exhibited abnormal high values in at least 1 year (see text for more details). All *P*-values can be found in Supporting information, Appendix S3

Figure 5. Population-normalized 11-nor-9-carboxy-delta9-tetrahydrocannabinol (THC-COOH) loads of a single 1-week period per year. See Table 1 for more information. **<LOQ**: concentrations in all daily samples were below limit of quantification (LOQ). **Grey dashed line**: 2013 overall mean of all participating cities. **Dot colour**: white: concentrations in all samples were above LOQ; Grey shading: one or more concentrations were below LOQ and set to 0.5*LOQ (the darker the grey, the more concentrations were below LOQ). **Numbers in brackets**: cities' rank (average over all available years). Cities in **bold type** participated in all 3 years and were used to calculate annual overall means (see Table 2). All *P*-values can be found in Supporting information, Appendix S3

Table 2. Population-weighted overall mean loads (units = mg/1000p/d). The loads in cities with all concentration values <LOQ were set to 0. Loads range from (close to) 0 up to several 10–100 mg/1000 person/day among cities, which implies large standard deviation (SD) or 95% confidence interval (CI) for all substances' overall means. Therefore, significance of changes cannot be meaningfully assessed for overall means and is assessed at cities' individual levels only (see Figs 1 –5 and Supporting information, Appendix S3)

	BE		MDMA		AMPH		METH		THC-COOH	
	a	b	a	b	a	b	a	b	a	b
2011 {14.12}	**249** [8.57]	311 [14.12]	***21*** [7.82]	21 [13.38]	**29** [8.12]	30 [13.67]	***31*** [7.51]	22 [13.07]	**71** [4.37]	69 [7.97]
2012 {11.50}	**254** [7.94]	229 [11.50]	**24** [7.19]	20 [10.75]	**29** [7.49]	32 [11.05]	**23** [6.89]	42 [11.50]	**60** [3.73	73 [9.07]
2013 {24.74}	**247** [8.77]	263 [24.74]	**25** [8.02]	18 [23.99]	**34** [8.32]	28 [24.20]	**17** [7.71]	33 [23.68]	**87** [4.53]	80 [15.55]

[a] Only cities participating in all 3 years are considered. These cities are labelled in bold type in the corresponding figures 1–5. Cities with 'explainable anomalies' for a particular substance are excluded from the calculation of overall means and labelled in italic type (even if the anomaly occurred only in 1 year). [b] All cities participating in the corresponding year are considered except the ones that were already excluded due to 'explainable anomalies' in option a. Cities with 'explainable anomalies' for a particular substance are excluded from the calculation of overall means and labelled in italic type (even if the anomaly occurred only in 1 year). { } Total population in millions monitored (please note: not all substances were measured in all cities). [ ] Population in millions contributing to the corresponding overall mean. BE = benzoylecgonine; MDMA = 3,4-methylenedioxy-methamphetamine; AMPH = amphetamine; METH = methamphetamine; THC-COOH = 11-nor-9-carboxy-delta9-tetrahydrocannabinol.

Benzoylecgonine

The highest weekly mean BE loads in the period 2011–13 were observed in wastewater from Amsterdam, Antwerp, London and Zurich and were between 400–850 mg/1000 people/day (**Fig. 1**). Loads were also relatively high (between 200–550 mg/1000

people/day) in Barcelona, Basel, Geneva, Utrecht and Eindhoven. The lowest values (<100 mg/1000 people/day) were observed in locations from northern, eastern and southern Europe. These results suggest a clear geographical difference in COC consumption, with higher use in western Europe. This is further demonstrated when BE loads in locations from Germany are evaluated. Loads in Dresden (eastern Germany) are negligible, similar to the amounts seen in the Czech Republic, while loads in Dortmund (western Germany) are comparable to the loads observed in the Belgian, Dutch and Swiss cities.

The overall population-weighted mean loads of BE for the 16 locations included in all 3 years were almost identical (**Table 2**). This suggests a stable use of COC in the investigated locations in the period 2011–13. Location-specific results from 2011, 2012 and 2013 are generally in agreement (**Fig. 1**); however, in some cases, variations occurred. An increase in BE loads from 2012 to 2013 was observed in the Belgian and Swiss locations, while a decrease was observed in two Dutch locations (Utrecht and Amsterdam).

Besides the high variation of mean BE loads observed across Europe, this study also highlights differences among locations within countries. Results from Belgium, Czech Republic, Germany, Serbia, Slovakia, Sweden and Switzerland suggest that the consumption of COC is lower in smaller towns compared to larger cities (**Table 1**, **Fig. 1**). Qualitatively, this is in agreement with studies investigating more locations within a country [17 - 22], although some of these rely on grab samples or single days only. The difference between Dresden and Dortmund, two cities of similar size, is attributable to their geographic location within Germany, as discussed previously.

The population-weighted mean COC consumption, calculated from BE loads (see Calculations), for locations included in all study years is similar between years and varies from 887 mg/1000 people/day in 2013 to 912 mg/1000 people/day in 2012. With 366 million people living in the urbanized regions of the European Union and a mean purity of 39% [standard deviation (SD) = 12%] [27, 28], a rough extrapolation would imply that

832 kg of street purity COC per day is consumed by the urbanized population in the European Union in 2013.

Amphetamine and methamphetamine

Because AMP is a urinary metabolite of METH and as AMP in wastewater could subsequently result from the use of METH, loads of both substances in wastewater have to be evaluated in parallel. Moreover, the use of certain prescription drugs, such as selegiline, may also result in traces of AMP and METH in wastewater following its metabolism; however, prescription rates indicate that any contribution would typically be <1% of the total AMP signal [24, 29]. The most frequent amphetamine-like substance detected in the majority of the investigated locations was AMP. The highest AMP loads were found in Belgium and the Netherlands, followed by locations in northern Europe and western Germany. The locations with the highest METH loads were found in the Czech Republic, Slovakia, eastern Germany and northern Europe, while the observed METH loads in the rest of the studied locations was low to even negligible (**Figs 2 and 3**). The presented results suggest an apparent geographical difference in the use of the amphetamine-like stimulants. The consumption of AMP is more widespread in western Europe, while the use of METH is clearly shown in northern Europe, Slovakia and Czech Republic. The German results confirm the aforementioned trend in the use of amphetamine-like substances. In Dülmen and Dortmund (West), relatively high AMP and negligible METH use was observed, while for Dresden (East, proximity to Czech Republic) the opposite was found.

The weighted mean of METH loads for the cities that were included in all study years declined by 45% from 2011 to 2013 (**Table 2**), due to some location-specific changes. For AMP, the weighted mean of the cities included in the 3 years is similar (**Table 2**). In contrast to BE loads, the difference in AMP and METH loads between smaller towns and bigger cities within a country is less clear.

MDMA

The highest loads of MDMA were found in western European locations, while locations in northern, eastern and southern Europe presented substantially lower MDMA loads

(**Fig. 4**). This pattern is comparable to BE and AMP, as demonstrated by the locations within Germany, with low MDMA loads in Dresden and higher loads in Dortmund.

The weighted mean of MDMA loads for the cities included in all 3 study years was stable (**Table 2**). No substantial changes in per capita MDMA loads between years for the individual locations were observed, with some exceptions (**Fig. 4**). The mass loads of MDMA from Eindhoven in 2012 and 2013 were much higher compared to 2011, and in Utrecht significantly higher loads for MDMA were observed in 2011 compared to 2012 and 2013. An explanation for these high loads in Utrecht (2011) and Eindhoven (2012) is most probably a release of unconsumed MDMA into the sewer system that was confirmed by specific enantiomeric profiling of the wastewater [30]. These outliers were not taken into account when assessing temporal changes. MDMA loads are generally higher in larger cities compared to smaller towns, as can be seen in different locations within Belgium, Finland, Germany, Serbia and Slovakia. A notable exception is St Gallen in Switzerland, which showed MDMA loads comparable to the larger city of Zurich.

THC-COOH

The determination of THC-COOH in wastewater poses some (pre-)analytical challenges, and as a result not all laboratories could report results for this THC metabolite. Furthermore, results from the performed inter-laboratory exercises revealed that participating laboratories that reported results for THC-COOH have comparable analytical methods (*Z*-scores within the limits), but because of some unknown pre-analytical losses, underestimations of the absolute amounts are probably made (de Voogt *et al*., unpublished). In the present study, however, this is not a real issue, because the focus lies on the relative comparison of THC-COOH loads.

In contrast to the other investigated substances, no clear geographical pattern could be observed for THC-COOH loads in the different European locations (**Fig. 5**). The values for Amsterdam were (expectedly) the highest, as Amsterdam is known for its coffee shops and because the Netherlands produces large amounts of herbal cannabis with a relatively high content of THC [31]. Also notable are the high loads observed in the city of Novi Sad, Serbia.

The weighted mean of THC-COOH loads for cities that were included in all 3 years showed some subtle variation, pointing out a variable cannabis use (amount or potency) between 2011 and 2013 (**Table 2**). No clear difference in THC-COOH loads between smaller towns and larger cities could be observed from the gathered data.

Discussion

Comparison of wastewater results with surveillance data

Europe has an established multi-indicator system for drug surveillance that is based on standardized demand and supply information, as well as research and intelligence sources [32]. Prevalence estimates are derived from a mixture of survey results and indirect statistical methods that try to estimate the unobserved cases from registers of observed drug users, such as treatment attendees or arrestees [33]. These methods can provide information on the main classes of users, the frequency and mode of use of a drug as well as on the purity of the substances available on the market, while WWA can provide objective and timely information on the total amount of a drug used in a specific area. These methods are highly complementary and, if used together, can substantially improve the quality of information on drug use patterns.

In terms of prevalence at the population level, the findings from WWA are broadly in agreement, with respect to relative drug use levels, with existing estimates, although they are not directly comparable. The wastewater data, however, highlight the need to consider the contribution of high and low prevalence areas in the estimates of total drug use within a population. Due to differences in demographics, the ranking of the city-based estimates reported in this study do not necessarily have to agree with national survey-based estimates. This points to the need to collect contextual information for a meaningful interpretation of wastewater data. Future monitoring campaigns should therefore (i) include more cities with different demographics within a country and (ii) evaluate monitoring design strategies to find an optimum among feasible logistics, sufficient quality control and representativeness for an entire year [34].

The spatiotemporal data on drug use data reported are largely, but not totally, in line with what is observed from surveys and other sources. The stable levels of COC

suggested by the presented wastewater data differs from other demand and supply data, which report a decline in COC use [35]. With WWA, it is currently not possible to differentiate between smaller number of people using larger amounts or vice versa, or even evaluating differences in consumption due to changes in purity. The analysis on METH and AMP accords with other data sources. The use of METH is long established in the Czech Republic, Slovakia and eastern Germany [36], and more recently supply-side data point to an increased use of METH elsewhere, especially in Scandinavian countries where it has, at times, displaced AMP. The situation appears quite dynamic and largely supply-side-driven. The wastewater data reported here accords with, and complements, the existing analysis of this situation.

For both MDMA and cannabis use, the picture is less clear. High levels of MDMA and THC-COOH might be expected in the Dutch cities sampled, but it is surprising that MDMA stands out so prominently with respect to some of the other European cities. The most recent supply-side data suggest that there is more MDMA available on the European market, and it is interesting to note that there is no evidence of this from the wastewater data reported here. The findings for THC-COOH in Amsterdam are not too surprising, as it is known for its large non-resident population using cannabis.

Uncertainty assessment

Details on estimating U_S can be found in [37, 38]. Applying the same scenario as in [25] —i.e. 1% of users in the population with two relevant, substance-related toilet flushes— results in a maximum of 20% for a daily value of U_S. An objective assessment of U_C was derived from inter-laboratory tests and does not exceed 30% (de Voogt *et al.*, unpublished). Operational accuracy of flow meters (U_F) still proves to be a challenge, and in this study was assumed conservatively to be 20% [39]. Despite advances in estimating U_P [40] it remains difficult to obtain a site-specific estimate, and in our study we assume 20% (RSD) as an average [25, 40]. A conservative estimate of overall uncertainty for a 7-day average based on WWA is approximately 46% (RSD) for all substances and locations (see Supporting information, Appendix S2 for more details). A sensitivity analysis reveals that reducing all four uncertainty components U_i by approximately one-quarter ($U_S \approx U_F \approx U_P \approx 15\%$, $U_C \approx 23\%$) has the same effect as trying

to eliminate only one U_i (e.g. $U_C \approx 0\%$); in both cases the overall uncertainty would be around 33%.

In areas with leaky sewers the results from WWA may tend towards an underestimation of actual illicit drug loads. A certain fraction of the wastewater and illicit drugs discharged from households may not arrive at the WWTP. Information on the potential amount of exfiltration can be found in **Table 1**. Furthermore, in cases where population size is estimated from nutrient loads in the wastewater stream, the population could be overestimated if industrial contributions are not properly subtracted. This would lead to an underestimation of population-normalized drug loads. In contrast, WWA results may tend towards an overestimation of population-normalized drug loads if the residential population only was used for normalization, but a net increase on workdays is effective due to commuters. This and additional information is provided in **Table 1** and Supporting information, Appendix S3 for further data interpretation.

Conclusions

By successfully increasing the number of participating cities to 42 in 2013 (2011: 19, 2012: 23), this is now the biggest application of WWA covering 24.74 million people. The wastewater from approximately 8 million people was analysed for BE, AMP, METH and MDMA during a 1-week period over 3 consecutive years (approximately 4 million for THC-COOH). As such, this study provides the most actual evidence for the quantification of spatial differences and temporal changes in the consumption of illicit drugs across European regions. Relatively stable loads for all investigated substances were observed, except for METH (apparent decline in 2012). In general, spatial differences were in agreement with surveillance data, where available. Wastewater analysis provides the possibility to collect, and report, measurements more quickly and regularly than is the current norm for national surveys. Wastewater analysis provides a unique opportunity to obtain near-real-time data on illicit drug use and for future comparison with other surveillance data, or particularly where such data are missing. Therefore, it should be considered for implementation on an annual or even more frequent basis. Systematically gathering information on catchment characteristics (sewer system and population) seems as indispensable as inter-laboratory tests for a meaningful comparison of wastewater data, which requires concerted efforts of numerous partners and disciplines.

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Supplementary information

Supplementary data to this article can be found, in the online version, at http://onlinelibrary.wiley.com/enhanced/doi/10.1111/add.12570/.

References

1. European Monitoring Centre for Drugs and Drug Addiction. Assessing Illicit Drugs in Sewage—Potential and Limitations of A New Monitoring Approach. Luxembourg: Office for Official Publications of the European Communities; 2008.
2. Griffiths P., Mounteney J. Drug trend monitoring. In: Miller P. G. , Strang J. , Miller P. M. , editors. Addiction Research Methods. Oxford, UK: Wiley-Blackwell; 2010, pp. 337–354.
3. Zuccato E., Chiabrando C., Castiglioni S., Bagnati R., Fanelli R. Estimating community drug abuse by wastewater analysis. Environ Health Perspect 2008; 116: 1027–1032.
4. van Nuijs A. L. N., Castiglioni S., Tarcomnicu I., Postigo C., de Alda M. L., Neels H. et al. Illicit drug consumption estimations derived from wastewater analysis: a critical review. Sci Total Environ 2011; 409: 3564–3577.
5. United Nations Office on Drugs and Crime (UNODC). The challenge of new psychoactive substances. UNODC, Vienna, 2013.
6. Karolak S., Nefau T., Bailly E., Solgadi A., Levi Y. Estimation of illicit consumption by wastewater analysis in Paris area. Forensic Sci Int 2010; 200: 153–160.
7. Metcalfe C., Tindale K., Li H., Rodayan A., Yargeau V. Illicit drugs in Canadian municipal wastewater and estimates of community drug use. Environ Pollut 2010; 158: 3179–3185.
8. Terzic S., Senta I., Ahel M. Illicit drugs in wastewater of the city of Zagreb (Croatia)—estimation of drug abuse in a transition country. Environ Pollut 2010; 158: 2686–2693.
9. Irvine R. J., Kostakis C., Felgate P. D., Jaehne E. J., Chen C., White J. M. Population drug use in Australia: a sewage analysis. Forensic Sci Int 2011; 210: 69–73.

10. Reid M. J., Langford K. H., Mørland J., Thomas K. V. Quantitative assessment of time dependent drug-use trends by the analysis of drugs and related metabolites in raw sewage. Drug Alcohol Depend 2011; 119: 179–186.

11. van Nuijs A. L. N., Mougel J.-F., Tarcomnicu I., Bervoets L., Blust R., Jorens P. G. et al. Sewage epidemiology—a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium. Environ Int 2011; 37: 612–621.

12. Bijlsma L., Emke E., Hernandez F., de Voogt P. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. Chemosphere 2012; 89: 1399–1406.

13. Harman C., Reid M., Thomas K. V. In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage. Environ Sci Technol 2011; 45: 5676–5682.

14. Lai F. Y., Bruno R., Hall W., Gartner C., Ort C., Kirkbride P. et al. Profiles of illicit drug use during annual key holiday and control periods in Australia: wastewater analysis in an urban, a semi-rural and a vacation area. Addiction 2013; 108: 556–565.

15. Zuccato E., Castiglioni S., Tettamanti M., Olandese R., Bagnati R., Melis M. et al. Changes in illicit drug consumption patterns in 2009 detected by wastewater analysis. Drug Alcohol Depend 2011; 118: 464–469.

16. Chen C., Kostakis C., Harpas P., Felgate P. D., Irvine R. J., White J. M. Marked decline in 3,4-methylenedioxymethamphetamine (MDMA) based on wastewater analysis. J Stud Alcohol Drugs 2011; 72: 737–740.

17. Huerta-Fontela M., Galceran M. T., Martin-Alonso J., Ventura F. Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain. Sci Total Environ 2008; 397: 31–40.

18. van Nuijs A. L. N., Pecceu B., Theunis L., Dubois N., Charlier C., Jorens P. G. et al. Can cocaine use be evaluated through analysis of wastewater? A nation-wide approach conducted in Belgium. Addiction 2009; 104: 734–741.

19. Kankaanpää A., Ariniemi K., Heinonen M., Kuoppasalmi K., Gunnar T. Use of illicit stimulant drugs in Finland: a wastewater study in ten major cities. Sci Total Environ; in press; 2014; doi:10.1016/j.scitotenv.2013.11.095

20. Vuori E., Happonen M., Gergov M., Nenonen T., Järvinen A., Ketola R. A. et al. Wastewater analysis reveals regional variability in exposure to abused drugs and opioids in Finland. Sci Total Environ; in press; 2014; doi:10.1016/j.scitotenv.2013.11.010

21. Östman M., Fick J., Näsström E., Lindberg R. H. A snapshot of illicit drug use in Sweden acquired through sewage water analysis. Sci Total Environ 2014; 472: 862–871.

22. Nefau T., Karolak S., Castillo L., Boireau V., Levi Y. Presence of illicit drugs and metabolites in influents and effluents of 25 sewage water treatment plants and map of drug consumption in France. Sci Total Environ 2013; 461–462: 712–722.

23. Banta-Green C. J., Field J. A., Chiaia A. C., Sudakin D. L., Power L., deMontigny L. The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal sewage. Addiction 2009; 104: 1874–1880.

24. Thomas K. V., Bijlsma L., Castiglioni S., Covaci A., Emke E., Grabic R. et al. Comparing illicit drug use in 19 European cities through sewage analysis. Sci Total Environ 2012; 432: 432–439.

25. Castiglioni S., Bijlsma L., Covaci A., Emke E., Hernandez F., Reid M. et al. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers. Environ Sci Technol 2013; 47: 1452–1460.

26. Lai F. Y., Ort C., Gartner C., Carter S., Prichard J., Kirkbride P. et al. Refining the estimation of illicit drug consumptions from wastewater analysis: co-analysis of prescription pharmaceuticals and uncertainty assessment. Water Res 2011; 45: 4437–4448.

27. United Nations, Department of Economic and Social Affairs, Population Division. World Urbanization Prospects: The 2011 Revision, CD-ROM edition. 2012. New York, NY.

28. European Monitoring Centre for Drugs and Drug Addiction. Purity of cocaine products at retail level, 2011. Available at: http://www.emcdda.europa.eu/stats13#display:/stats13/ppptab7a (accessed 12 December 2013).

29. Baker D. R., Barron L., Kasprzyk-Hordern B. Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: chemical analysis and drug use estimates. Sci Total Environ; in press; 2014; doi:10.1016/j.scitotenv.2013.11.107

30. Emke E., Evans S., Kasprzyk-Hordern B., de Voogt P. Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: a Dutch perspective. Sci Total Environ; in press; 2014; doi:10.1016/j.scitotenv.2013.11.043

31. European Monitoring Centre for Drugs and Drug Addiction. EU Drug Market Reports—A Strategic Analysis. Luxembourg: Office for Official Publications of the European Communities; 2013.

32. Griffiths P., Mounteney J., Lopez D., Zobel F., Götz W. Addiction research centres and the nurturing of creativity. Monitoring the European drug situation: the ongoing challenge for the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Addiction 2012; 107: 254–258.

33. Frischer M., Hickman M., Kraus L., Mariani F., Wiessing L. A comparison of different methods for estimating the prevalence of problematic drug misuse in Great Britain. Addiction 2001; 96: 1465–1476.

34. Ort C., Eppler J. M., Scheidegger A., Rieckermann J., Kinzig M., Sörgel F. Challenges of surveying wastewater drug loads of small populations and generalizable aspects on optimizing monitoring design. Addiction 2014; 109: 472–481.

35. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2013: Trends and Developments. Luxembourg: Office for Official Publications of the European Communities; 2013.

36. Pietsch J., Paulick T., Schulz K., Flössel U., Engel A., Schmitter S. et al. Escalation of methamphetamine-related crime and fatalities in the Dresden region, Germany, between 2005 and 2011. Forensic Sci Int 2013; 233: 51–54.

37. Ort C., Lawrence M. G., Reungoat J., Mueller J. F. Sampling for PPCPs in wastewater systems: comparison of different sampling modes and optimization strategies. Environ Sci Technol 2010; 44: 6289–6296.

38. Ort C., Lawrence M. G., Rieckermann J., Joss A. Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review. Environ Sci Technol 2010; 44: 6024–6035.

39. Thomann M. Quality evaluation methods for wastewater treatment plant data. Water Sci Technol 2008; 57: 1601–1609.

40. O'Brien J. W., Thai P. K., Eaglesham G., Ort C., Scheidegger A., Carter S. et al. A model to estimate the population contributing to the wastewater using samples collected on census day. Environ Sci Technol 2014; 48: 517–525.

Chapter 6.3.4, scientific monograph, chapter 2

Estimating community drug use through wastewater analysis

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Chapter 2 in "update of the 2008 insights on: assessing illicit drugs in wastewater - potential and limitation of a new monitoring approach". EMCDDA insight (2014) submitted

Introduction

The wastewater-based epidemiology approach relies on the principle that traces of almost everything we consume are excreted unchanged or as a mixture of metabolites in urine and/or faeces and ultimately ends up in the sewer network. Thus, measuring target drug metabolic residues in raw wastewater allows identifying the use of specific substances in a population.

Up to date, the most popular application of this approach is for estimating illicit drugs use in a community. The method consists of several consecutive steps allowing researchers to identify and quantify target metabolic residues of illicit drugs in raw wastewater and to back-calculate the amount of the corresponding illicit drugs that has been consumed by a population served by a sewage treatment plant (STP). A general scheme of the approach is reported in **Figure 1**. Firstly, representative composite samples of raw wastewater should be collected and analysed for the selected substances. The back-calculation of drug consumption is performed by i) calculating daily sewer loads of target residues (µg/day) by multiplying concentrations of measured target residues (ng/L) with daily flow rates of sewage (m^3/day), ii) estimating total consumption by applying specific correction factor, which consider the average excretion rate of a given drug residue and the molecular mass ratio of parent drug/metabolite (Zuccato et al., 2008; van Nuijs et al., 2011), iii) normalizing consumption by dividing daily values by number of people to facilitate comparison among cities (mg/day/1000people) and iv) assuming a mean dose to obtain a result in doses/day/1000people.

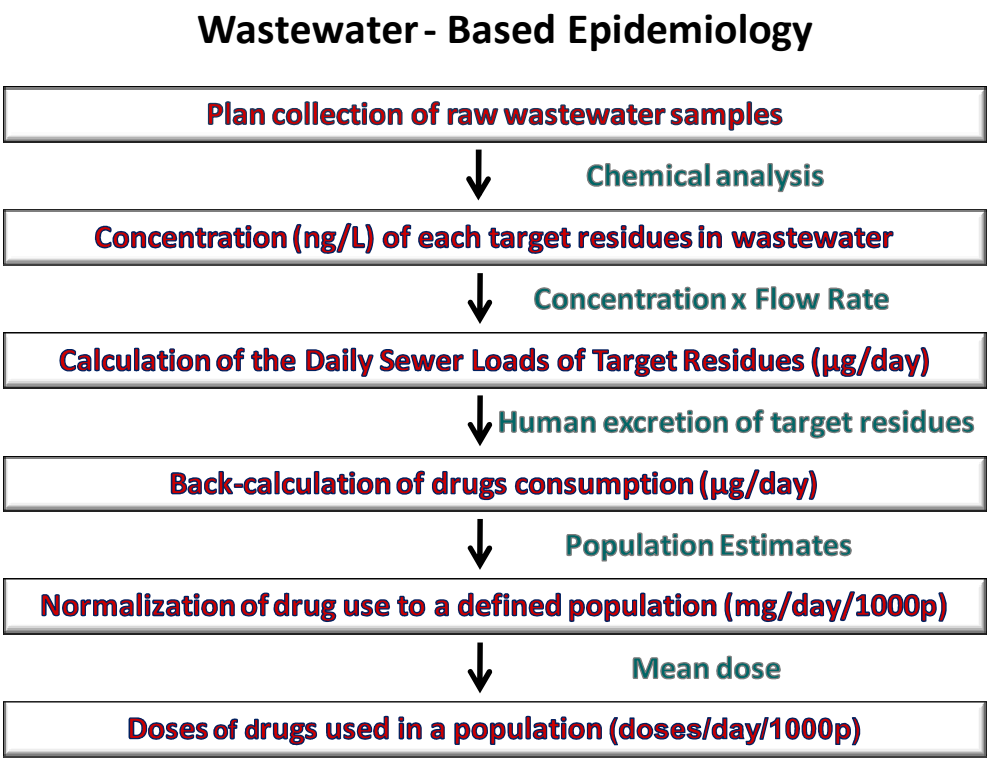


Figure 1 Main consecutive steps of the wastewater analysis approach and data required for each step (Modified from Castiglioni et al., 2013b).

Between 2005 and 2010, an increasing number of research groups have applied their own methods to assess the use of illicit drugs on local and national scales in several countries demonstrating the potential of the approach for quantifying illicit drug use at a community level. Unfortunately, it was somewhat difficult to compare the results of these early studies because of the lack of a common procedure ensuring comparable approaches for the sampling of wastewater and for the back-calculation of illicit drug consumption. It became therefore urgent to establish some practical guidelines to ensure a proper application of the approach. In 2010, a group of researchers working in this field established the Sewage analysis CORe group Europe (SCORE) network to harmonize the wastewater-based epidemiology approach and to coordinate international studies through the establishment of a common protocol of action. The first activity organized by the SCORE group was a Europe-wide investigation performed

in 2011 in 19 European cities which allowed the first ever wastewater study on the regional differences in illicit drug use in Europe (Thomas et al., 2012). This study included also the first intercalibration exercise to evaluate the quality of the analytical data and allowed also a comprehensive characterization of the major uncertainties of the approach (Castiglioni et al., 2013).

This chapter will provide an overview of the wastewater based epidemiology approach byl describing the best-practice protocol established during this first European monitoring study which represents the most comprehensive information available on this topic including the latest improvements to reduce the uncertainty associated to the approach. Research updates on sampling, chemical analyses, stability of target residues in wastewater and population size estimation will be provided. Moreover, new analytical techniques such as the analysis of enantiomers of chiral compounds, which allows distinguishing the amounts of drugs consumed from those discharged in urban wastewater, will be described. Chapter 3 will deal specifically with the choice of suitable drug target residues (biomarkers) and the development of reliable back-calculation methods.

Best practice protocol

The main aims to establish a best-practice protocol were: 1) produce homogeneous and comparable data at different sites and 2) provide the most reliable estimates of drug use to consistently complement existing epidemiological studies. In view of the enormous potential of the wastewater-based epidemiology approach and of its wide application by different research groups, it is now highly recommended for all the groups working in this field to follow a common procedure while implementing the approach locally.

The best-practice protocol consists of several guidelines addressing samples collection, storage and chemical analyses. A summary of the main points is reported in **Table 1** and the entire protocol is available at http://www.emcdda.europa.eu/wastewater-analysis. The protocol was established and formally agreed at a meeting held at Dublin City University, (Ireland) on the 14th of December 2010. Later on the protocol was

revised and improved following new expertise gained during the successive analytical campaigns conducted in Europe. On one side, sewer engineers were involved to evaluate the influence of different sewer designs and sampling procedures on the data generated. On the other side, analytical chemists were involved into establishing common procedures to evaluate the quality of analytical results and identify the best conditions for samples handling during storage and analyses.

Table 1 Summary of the main procedures reported in the best practice protocol currently adopted in Europe-wide studies.

Phase of the approach	**Agreed procedures**
Sampling and samples handling	*Sampling point:* STP influent *Sample Type*: 24-h flow-weighted composite *Sampling container:* PET or glass container *Questionnaire:* developed to collect information on sewer systems, sampling mode and additional parameters such as BOD, COD, N, P, flow data, type of sewage influent, temperature, pH.
Storage treatment during sampling	*During sampling:* <4 °C *After sampling - two possible options:* 1. Process the sample for analyses within 12h 2. Freeze the samples immediately after collection
Chemical analysis –Quality control	*Substances investigated:* cocaine, benzoyl-ecgonine, amphetamine, methamphetamine, ecstasy (3,4-methylenedioxymethamphetamine-MDMA), 11-nor-9-carboxy-delta9-tetrahydro-cannabinol (THC-COOH) *Internal quality control:* Use of labeled analytical standards for each compound *External quality control:* Analysis of methanol standards and influent samples as prepared by one laboratory

The established common protocol of action tested during the first European study, was later adopted over two successive studies conducted in 2012 in 25 cities and in 2013 in 43 cities (Ort et al., 2014a). The concerted effort to produce comparable results allowed the provision of the most actual information on illicit drug use in Europe and the first ever quantitative measurements of illicit drug use in certain European countries.

Optimization of sampling and monitoring, challenges and alternatives

The first step to estimate drug use through wastewater analysis is the collection of "representative samples", that should contain the entire amount of a substance discharged into wastewater daily from a defined community. Proper procedures should be therefore adopted to collect such samples from untreated wastewater at the entrance of STPs. In general terms, decreasing cost of incorrect decisions requires a reduction of uncertainty. This in turn implies an increase of costs for measurements, i.e. number of samples and effort for chemical analysis. Consequently, an optimum must be found, the so-called fit-for-purpose uncertainty (Ramsey and Thompson, 2007). How does this look like in wastewater-based epidemiology? This may be difficult to answer without an estimate for expected cost of incorrect decisions.

It seems important to note that wastewater-based epidemiology intends to build on existing infrastructure and samples come at almost no cost (Banta-Green and Field, 2011), except for logistics. Therefore no relevant changes occurred related to the employed sampling techniques since the publication of the last issue of EMCDDA Insights 9 on wastewater (Rieckermann, 2008). Hence, the aim of this section is to elucidate on scientific advancements since 2008 to answer the following three questions: 1) Which level of uncertainty can be achieved with the sampling equipment in place and routinely applied sampling modes and frequencies? 2) Are there situations that require particular attention? 3) Are there alternative sampling technologies applicable to raw wastewater?

Fluctuations of illicit drugs in sewers

The statement that "almost everything that is worth analysing is actually or potentially heterogeneous." (Thompson, 1999) also holds true for illicit drugs in sewers. Targeted

high-frequency sampling campaigns have revealed high temporal fluctuations of concentrations of illicit drugs and pharmaceuticals, caused by toilet flushes containing these substances or pump stations lifting and transporting wastewater from entire sub-catchments intermittently to STPs. Specifically-tailored sampling proficiency tests demonstrated that inadequate sampling modes (e.g. grab samples or time-proportional composite sampling) and frequencies (i.e. intervals longer than 1h) can lead to substantial sampling artifacts which can result in both over or underestimation of results. In these cases sampling errors can be larger than errors due to chemical analysis (Ort et al., 2010a, Ort et al., 2010b).

Collecting 24-h composite samples

For numerous practical reasons, normally 24-h composite samples of raw wastewater at the influent of STPs are collected (Ort, 2014b). As such, daily samples are the unit for analysis. Studies focusing on relatively large catchments and relatively frequently used substances conclude that sampling uncertainty can be kept below 10% (RSD) (Mathieu et al., 2011; Thomas et al., 2012; Castiglioni et al., 2013). Due to systematic diurnal variations of wastewater flows and drug loads (Brewer et al., 2012; Lai et al., 2013) samples need to be collected in flow-or volume-proportional manner (Ort et al., 2010a, Ort et al., 2010b) to avoid incorrectly weighted samples and biased results. Furthermore, due to potential short-term fluctuations, it is recommended to apply sampling intervals not exceeding 5-10minutes. This result in approximately 100-200 individual samples collected over a 24-h period. It should be noted that all these samples are pooled to one sample before analysis and that a high sampling frequency does not necessarily increase analytical effort. A questionnaire to aid in collecting relevant details to estimate or minimize sampling uncertainty in wastewater-based epidemiology is provided in the Supporting Information of (Castiglioni et al., 2013), and a free open source software can be found on www.eawag.ch/spg.

Estimating annual averages

An annual average cannot be directly observed by analysing few samples, but it needs to be estimated from a sufficient number of 24-h composite samples collected throughout a year. This number depends highly on the weekly and seasonal variation –

which is typically unknown – and the desired level of accuracy. To date, only five studies investigated daily loads of illicit drugs over a one-month period or more summarized in (Ort et al., 2014c). **Figure 2** shows the observed variations of daily drug loads expressed as coefficient of variation (CV). For benzoylecgonine, measured in all studies, load variations decrease with increasing population size. For other substances such an expected decrease could not be confirmed for various reasons. The high variation of 3,4-methylenedioxymethamphetamine (MDMA) loads is mainly attributed to regularly high consumption on weekends and fairly low consumption during working days (or non-detects). The required number (n) of samples to stay below a certain level of uncertainty (U) can be calculated according to $n=(CV/U)^2$. The laborious task is to obtain site- and substance-specific CVs. Based on the limited data available to date, it seems as if a CV of 75% was only exceeded in rare cases and that one could take this as a reasonable value. For certain substances CVs can also be substantially lower, which would imply a smaller number of required samples for the same level of accuracy. However, one needs to keep in mind that most samples are analyzed for multiple substances and that the substance with the highest CV dictates the number of samples. Should the uncertainty of an annual mean not exceed 20%, one would need 14 samples randomly distributed over a year (or 56 samples for U=10%).

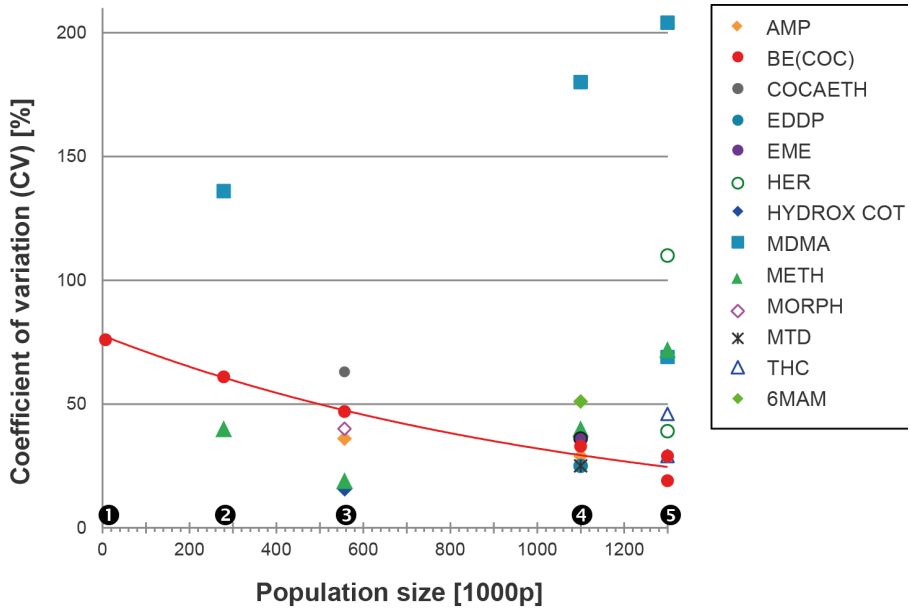


Figure 2 Variability of daily drug loads expressed as coefficient of variation (CV = standard deviation / mean) for five long-term studies. Population sizes (P) and number of subsequent monitoring days (d) for the five studies are ❶ P=7160, d=1369; ❷ P=278'000, d=336; ❸ P=557'000, d=28; ❹ P=1.1million, d=336; ❺ P=1.3million, d=28 and 35 (references and details in (Ort et al., 2014c).

Legend: AMP=amphetamine; BE=benzoylecgonine; COC=cocaine; COCAETH= cocaethylene; EDDP=2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME= ecgonine methyl ester; HER= heroin; HYDROX COT= hydroxycotinine; MDMA= 3,4-methylenedioxymethamphetamine; METH= methamphetamine; MORPH= morphine; MTD= methadone; THC= Δ9-tetrahydrocannabinol; 6MAM= 6-monoacethyl-morphine.

Challenges and alternatives

Wastewater-based epidemiology in the future may also require sampling from small STPs which are often not equipped with sampling devices to collect raw influent wastewater. Furthermore, wastewater concentrations of illicit drugs and flows from small catchments can be subject to much higher fluctuations, which requires flow-or volume-proportional sampling at even higher frequencies than in the influents of large STPs (i.e. down to 1-5 min). This is even more pronounced in the effluent of individual premises, such as schools or prisons. Another challenge is assessing the accuracy of flow

measurements (Rieckermann, 2008). This can be partly resolved when the population size to calculate population-normalized drug loads is estimated from wastewater parameters (Lai et al., 2011, O'Brien et al., 2014,) (see also chapter on *population estimation*). An alternative technology to active sampling is passive sampling. This involves the placement of a device (passive sampler) in the wastewater where it accumulates chemicals by diffusive processes in an integrative manner over time. Such technologies offer practical and economic advantages for gathering long term, or geographically broad data. For example they have been used to estimate drug use in Oslo Norway, for a period of one year (Harman et al., 2011). It should be noted that there are several challenges involved with applying these techniques, including calibration and quantification, knowledge of kinetics, and correction for different exposure scenarios (Harman et al., 2012).

Wastewater-based epidemiology has to "fear the sampling dogs" [1] only in specific settings where target residues' dynamics are extraordinary high because of 1) a small absolute number of wastewater pulses containing the substances of interest (searching the needle in the hay stack) and 2)sampling locations close to the source. The latter is the case in the effluent of individual premises or influents to small STPs. This is because toilet flushes are not attenuated by dispersion effects to the same extent over short travel distances than by longer travel distances. A toilet flush directly outside a house may only extend over a couple of seconds, depending on the sanitary installations and hydraulic conditions in the house connection. For high prevalence drugs in large catchments, current best practice in sampling is expected to result in uncertainties that are smaller or in the same range as other components of uncertainty (Castiglioni et al., 2013; Ort et al., 2014c).

Chemical analysis and quality control

The estimation of community drug use through wastewater analysis requires accurate and sensitive quantification of illicit drug target residues (commonly the unaltered drug

[1] " 'How reliable are these analytical results?' […] there is an understandable lack of enthusiasm for rousing the sleeping dogs of sampling when there is a fair chance of being severely bitten."

or the main metabolite excreted in urine). Reliable data are the basis of subsequent calculations of drug loads in wastewater and consumption. The principal difficulty for the quantitative analysis of illicit drugs deals with their very low concentrations in combination with the complexity and unknown composition of wastewater. Concentrations of illicit drugs in wastewaters are generally around a factor 1000 lower than in human biological fluids. Furthermore, the presence of a large number of other substances in the sample matrix may hamper sensitive quantification and reliable identification. Hence, proper detection technologies should be specific and sensitive enough at the same time.

Modern analytical chemistry offers the solution to this challenging task. The use of advanced analytical techniques and the expertise of the analyst are essential to obtain accurate data for drug residues in wastewater at trace level (ng/L) (Castiglioni et al., 2008, Postigo et al., 2008a). The medium-high polarity and commonly low volatility of these compounds made liquid chromatography coupled to mass spectrometry (LC-MS) the technique of choice, particularly when using tandem mass spectrometry (MS/MS) (Castiglioni et al., 2013). LC-MS/MS allows simultaneous quantification and identification of the target compounds in complex matrices thanks to its excellent sensitivity and selectivity. The substances are ionized, fragmented and detected by monitoring specific m/z (mass/charge) ions for each compound. Typically, two transitions are acquired, by selecting the precursor ions and product ions/fragments characteristic of the compound under study: one, commonly the most intense, for quantification (Q), and another for confirmation (q). As an example, **Figure 3** illustrates the detection and identification of cocaine and its main metabolite benzoylecgonine on the basis of two transitions acquired for each compound (Q, q). For cocaine, the specific fragments m/z 182 and m/z 82, from the precursor ion m/z 304, were selected (left panel), and for benzoylecgonine the specific fragments m/z 168 and m/z 82, from the precursor ion m/z 290, were used (right panel). Considering the peak area of the quantitative transition (Q) in the sample and comparing it to that obtained in the reference standard it is possible to calculate the concentration of each substance. The acquisition of two transitions, together with retention time data and the measurement of ion intensity ratios between recorded transitions in standards and samples, permits a

reliable identification of the compound detected, even at very low concentrations (see Q/q ratios in the figure and deviation, which is within the maximum tolerance admitted) (United Nation Industrial Development Organization, 2006).

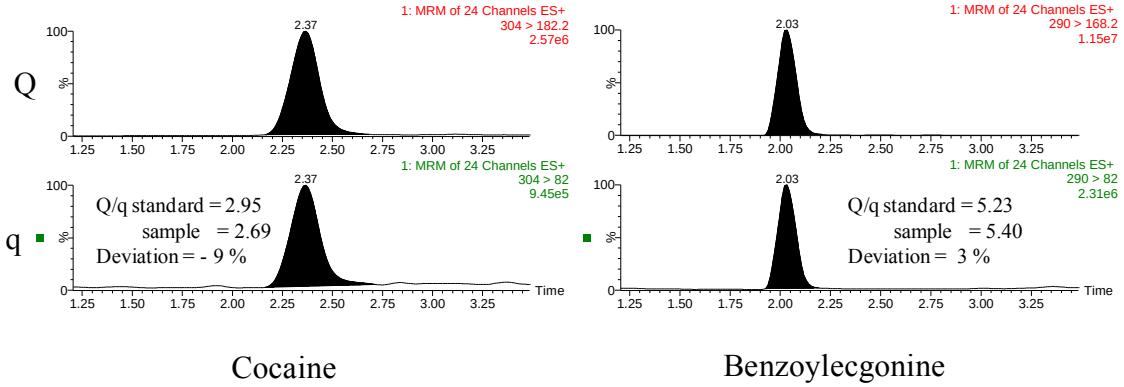


Figure 3 Identification and quantification of cocaine (382 ng/L) and its major metabolite benzoylecgonine (931 ng/L) in wastewater. (Q: quantification transition, q: confirmation transition).

Despite the strong potential of LC-MS/MS, other compounds present in the sample may interfere and compete with the target residues in the ionization process (matrix effects). The removal, minimization or correction of matrix effects is one of the key aspects within the analytical methodology to ensure accurate quantification and reliable identification. Although the sensitivity of modern instrumentation is excellent, it is necessary to apply a sample treatment step, which main objective is to pre-concentrate the analytes and perform a clean-up of the sample. Solid-phase extraction (SPE) is widely applied to this aim. Other alternative sample treatments, such as on-line SPE (Postigo et al., 2008a) and large volume injection (Chiaia et al., 2008; Berset et al., 2010) open the possibility for a full-automated analysis. In the near future, new and even more sensitive LC-MS/MS instruments may help to improve the performance of the methods, and will allow an extra dilution of samples extracts or lower pre-concentration factors, helping to minimize matrix effects.

Most of the reported methodologies use internal standards, which are added to the samples as surrogates (i.e. previously to sample treatment) for more accurate quantification. Reference standards , preferably the isotope-labeled analyte for each target compound, are commonly added to compensate for matrix effects and to ensure the satisfactory correction for analytical errors associated to sample manipulation and storage.

Nowadays, LC-MS/MS has wide recognition and acceptance for accurate quantification of target drug residues in wastewater. However, high resolution mass spectrometry (HRMS) opens new perspectives in this analytical field due to the powerful information provided by this technique (accurate-mass full-spectrum mass data). When using LC-MS/MS, the determination is directed towards specific compounds that have been previously selected, and is therefore limited to those substances for which the method has been developed. Consequently, other compounds different than target compounds may be ignored in the analyses. HRMS transcends this limitation and shows strong potential for target and non-target screening of large number of compounds. Investigation of transformation products that can be formed in water is also one of the main possibilities offered by this technique. LC-HRMS is commonly limited to qualitative screening, i.e. detection and identification, but recent improvements promoted its use for accurate quantification too (Gonzalez-Marino et al., 2012; Bijlsma et al., 2013).

Any analytical methodology should comply with strict quality requirements in order to generate reliable data. Quantitative method validation is obviously required, but the application of updated criteria based on the acquisition of several transitions, considering their specificity, or based on mass accuracy measurement is also necessary. Furthermore, the analysis of internal quality controls in each sample sequence ensures quality and test for daily method variations. A key aspect is to generate data comparable between different laboratories. This makes the performance of inter-laboratory exercises, where the same sample is analyzed by all participants, necessary. The results obtained provide an indication of the accuracy and performance of each laboratory and the presence (or absence) of systematic errors.

Most research in this field has aimed until now at estimating the use of the 'classical' illicit drugs (amphetamine, cannabis, cocaine, ecstasy and methamphetamine). However, advanced analytical techniques currently available have allowed investigating the presence of other compounds in wastewater, such as new psychoactive substances (NPS) which continuously appear on the market (Reid et al., 2013;van Nuijs et al., 2013). To this aim, non-target HRMS can especially be very attractive, due to the lack of reference standards in many cases, or the lack of available information on the metabolism of new psychoactive substances (Ibáñez et al., 2014).

Enantiomeric profiling of illicit drugs

When the presence of illicit drugs in wastewater is monitored regularly using frequent sampling intervals a sort of baseline of daily drug loads as a result of consumption in the corresponding community can be estimated. In some cases, however, aberrantly high loads may be observed in the sewer which cannot possibly correspond to the actual drugs consumed in that specific community. These abnormally high loads may result from direct disposal of unused drugs and/or production waste from, e.g., illegal manufacturing facilities, making the epidemiological estimation of community-wide drug use via wastewater analysis difficult and potentially unreliable. It is therefore of the utmost importance to introduce new approaches for making a distinction between consumption and direct disposal of unused drugs to wastewater (Emke et al., 2013). Enantiomeric profiling of drugs in wastewater by chiral chromatography coupled with mass spectrometry could be a viable option to solve the above mentioned problems.

A chiral molecule usually has at least one chiral centre (e.g. asymmetric carbon) as a result of which it shows optical activity. It exists in the form of two enantiomers (if only one chiral centre is present), being the non-superimposable mirror images of each other (**Figure 4**) (Evans and Kasprzyk-Hordern, 2014). Many of the popular psychoactive illicit drugs and new designer drugs (e.g., cocaine, amphetamines, cathinones) contain one or more asymmetric carbon atoms (Emke et al., 2013). The enantiomers of the same compound exhibit the same physiochemical properties but they differ in their

biological properties. The distribution in the body, metabolism and excretion from the body favors one enantiomer over the other. This results from the fact that enantiomers stereoselectively react in biological systems, e.g. with enzymes (Kasprzyk-Hordern, 2010). Two enantiomers of the same drug can also exhibit different potencies, e.g. S(+)-3,4-methylenedioxymethamphetamine (MDMA) is known to be more amphetamine-like and R(-)-MDMA is known to be more hallucinogenic; S(+)-amphetamine has twice as high stimulant activity than R(-)-amphetamine (Kasprzyk-Hordern and Baker, 2012a). The chemical synthesis of compounds containing one asymmetric centre will generally lead to equal amounts of the two corresponding enantiomers (a racemic mixture) in the product synthesized (e.g. synthesis of MDMA). The ratio of the concentration of one enantiomer to the sum of both R(-) and S(+) forms: R(-) / [R(-) + S(+)], can be defined as the Enantiomeric Fraction (EF), a racemic mixture will therefore have a EF of 0.5. It should be however emphasized that certain illicit drugs are synthesized via stereoselective routes (subject to availability of substrates). For example, more potent S(+)-methamphetamine is usually synthesized in clandestine laboratories by reduction of 1R,2S(-)-ephedrine or 1S,2S(+)-pseudoephedrine (naturally produced by ephedra) (Kasprzyk-Hordern and Baker, 2012a).

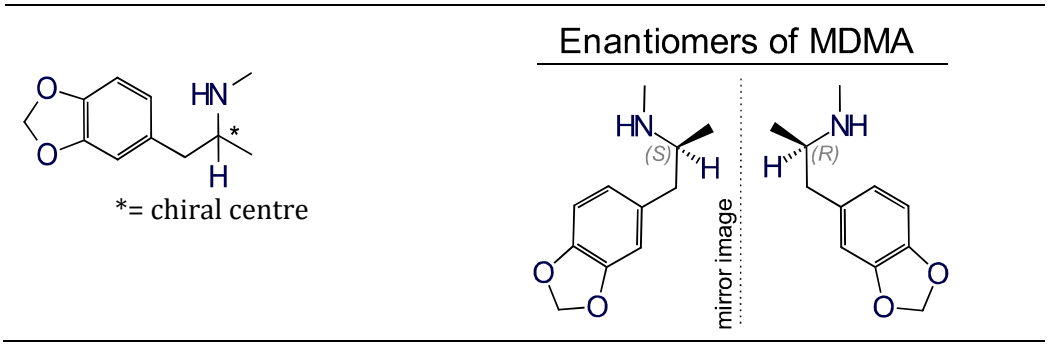


Figure 4 Enantiomers of 3,4-methylenedioxymethamphetamine (MDMA).

Human metabolism of a product containing a racemic mixture of the enantiomers will change the enantiomeric ratio as a result of differences in metabolic conversion rates of the enantiomers (Emke et al., 2013). For example: S(+)-amphetamine is preferentially metabolized leading to enrichment of amphetamine excreted with urine with R(-)enantiomer (Kasprzyk-Hordern and Baker, 2012a). Furthermore the enantiomeric ratio

can be influenced by microbial activity during sewage water transport in the catchment area and also by active sludge in the sewage water treatment plant leading to, for example, further enrichment of amphetamine and MDMA with R(-)-enantiomer (Kasprzyk-Hordern and Baker, 2012b).

Enantiomeric profiling of MDMA in wastewater

Many synthetic routes for producing MDMA start with piperonylmethyl ketone (PMK) and use either the Leuckart route or various reductive aminations (Renton et al., 1993). All of these methods produce racemic MDMA. S(+)-MDMA is however known to undergo preferential metabolism over R(−)-MDMA, which leads to enrichment of MDMA with R(−)-enantiomer and preferential formation of S(+)-3,4-methylenedioxyamphetamine (MDA) (Moore et al., 1996). Moore et al. observed also that both primary routes of excretion in human (bile and urine) had greater concentrations of R(−)-MDMA than the S(+)-enantiomer (EF of 0.57, autopsy findings). These fluids also contained twice the concentration of S(+)-MDA than the R(−)-enantiomer (EF = 0.37, autopsy findings). This is very important information, which allows for the verification of whether residues from a chiral drug present in wastewater result from its actual consumption (EF ≠ 0.5) or direct disposal (EF = 0.5). As MDMA does not currently have medical applications its presence in biological specimen is believed to result from its abuse (Emke et al., 2013). Indeed, Kasprzyk-Hordern and Baker (Kasprzyk-Hordern and Baker, 2012a) reported, in a first study of this kind, that MDMA was enriched with R(-)-enantiomer due to preferential metabolism of S(+)-MDMA in humans. Furthermore, the identified MDA was enriched with S(+)-enantiomer, which suggests that its presence might be associated with MDMA consumption and its subsequent metabolism into S(+)-MDA and not intentional MDA use (if the latter was true, MDA in wastewater would be enriched with R(-)-enantiomer).

In 2011 aberrantly high mass loads of MDMA were observed in the wastewater of the city of Utrecht in the Netherlands. These loads were highly deviating from the results observed in the previous monitoring campaign in 2010 (Bijlsma et al., 2012). Therefore enantiomeric profiling of these sewage water samples was undertaken to determine the origin of the illicit drugs. It was shown (**Figure 5**) that the average EF of MDMA was

0.54 for the sampling week in 2011. This shows that MDMA quantified in wastewater throughout the sampling week was racemic, which indicates its direct disposal in the sewage system and further explains high loads of MDMA quantified in Utrecht wastewater during the sampling week in 2011 (average load was 20 times higher than in 2010). The relatively slow decrease of the MDMA load shortly after the assumed disposal (red line in **Fig. 5**) can be explained by the characteristics of the STP in Utrecht where the effluent is partly recirculated (1/3 on a dry day) into the influent. This direct disposal could be the result of a police raid into an illegal production facility that took place two days before the monitoring started. The police estimated that 30 kg of raw MDMA or tablets had been disposed under the pressure of the police raid. In contrast, the samples from 2010 (green line in Fig. 5) showed an average EF of 0.65 corresponding to excretion profiles in urine after consumption of MDMA (Emke et al., 2013).

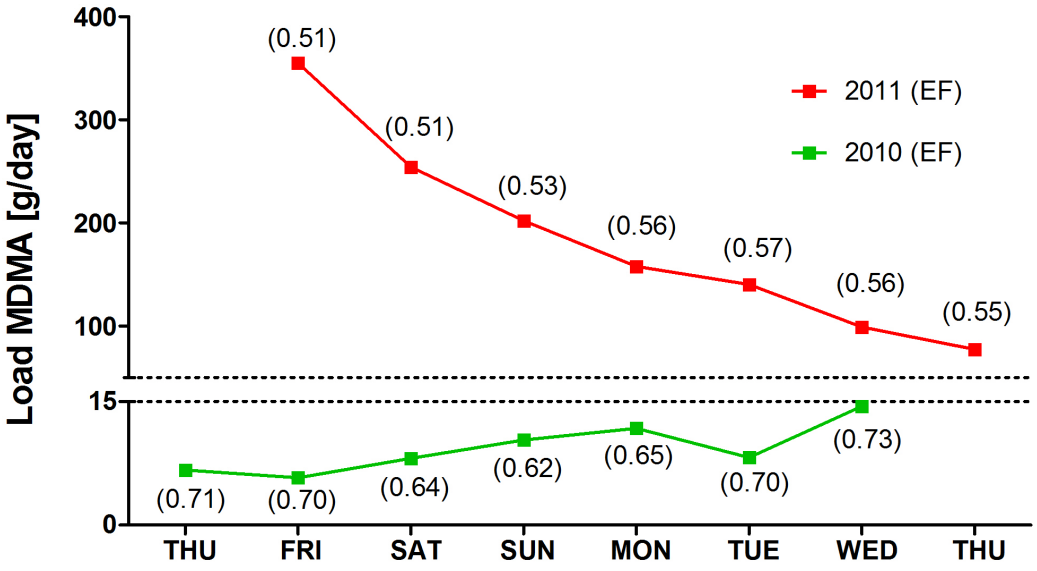


Figure 5 MDMA loads during two separate weeks sampled in 2010 and 2011 in the sewage treatment plant of Utrecht, the Netherlands and their corresponding enantiomeric fractions (EF).

Until now, it was difficult to determine if mass loads of studied drugs were actually originating from consumption, disposal of unused drugs or production waste. This uncertainty in the estimation of community wide drugs use should not be underestimated. In this framework, enantiomeric profiling of wastewater is a new and very promising approach to solve this problem.

Stability of drug residues in urban wastewater

Stability of drug residues in wastewater is a property that has to be evaluated with care, as it can lead to significant under-or overestimations in the calculation of drug use in wastewater-based epidemiology. Therefore, it is imperative to gather knowledge on the behavior of the target drug residues in the sewer system (from place of excretion to sample collection: in-sewer stability) and the stability of these compounds in the matrix during collection and storage of wastewater.

Table 2 gives an overview of the reported experiments which have assessed the transformation of drug residues in wastewater from the place of excretion to the sample collection in the wastewater treatment plant (in-sewer biotransformation). In all studies, the amphetamine-like stimulants under investigation were amphetamine, methamphetamine and MDMA and showed negligible transformation in wastewater after 12 h (or even up to 24 h) at room temperature. When the temperature of the wastewater used for the experiments was lowered to 4 °C, these amphetamine-like stimulants were stable up to 3 days. These observations are in agreement with stability studies for these three compounds in urine at 37 °C for 3 days and longer. Stability experiments in wastewater have been also performed for cocaine and its major metabolites, benzoylecgonine and ecgonine methyl ester. Benzoylecgonine was the most stable residue in wastewater, with less than 20% biotransformation after 24 h, at pH 7.5 and room temperature (**Table 2**). The observed increase in benzoylecgonine concentrations over the course of time were due to a partial degradation (hydrolysis) of cocaine to benzoylecgonine, a process which was also observed in blood and urine. The two other residues under investigation, cocaine and ecgonine methyl ester were significantly less stable in wastewater with losses up to 60% and 40%, respectively, after 12 h, at pH 7.5 and room temperature. However, some inconsistencies in the

degradation rates of these two compounds were observed between various studies, probably due to differences in the experimental setups such as the matrix (different characteristics of used wastewater), and spiking concentrations. Stability experiments for THC-COOH, the most abundant wastewater residue resulting from cannabis use, demonstrate that this compound is stable at relevant conditions (12 h, pH 7.5, and 20 °C). On the contrary, significant losses were observed in these conditions for the exclusive transformation product of heroin consumption, 6-monoacetylmorphine.

Considering typical in-sewer residence times are < 10 h, this means that transformation (or degradation) is generally lower than 10% for amphetamine, methamphetamine, MDMA, benzoylecgonine, and THC-COOH. In-sewer degradation has therefore negligible influence on wastewater analysis results when these compounds are used in back-calculations. However, when 6-monoacetyl-morphine is used for heroin back-calculations, underestimations of the actual heroin use are most probably made due to significant losses during in-sewer transformation.

Clearly, better designed and more sophisticated research in this area is necessary to assess other factors that can influence in-sewer losses and transformation, such as adsorption to solid matter, formation of biofilms and deconjugation processes. Moreover, most of these experiments have only been conducted in the laboratory mimicking "real conditions" for temperature and sewage composition. Only one modelling study addressing wastewater drug stability has been conducted to date (Plosz et al., 2013), thus it is highly required to design in sewer experiments and additional modelling studies to investigate in-sewer biotrasformation of target residues and to confirm the current information.

Besides the in-sewer transformation, it is important to evaluate the stability of drug residues in wastewater during sampling (typically 24-h composite) and during sample storage. The typical sampling strategy is in most cases performed at +4 °C (**Table 1**). Experiments summarized in **Table 2** have demonstrated that cocaine, ecgonine methyl ester, and 6-monoacetylmorphine are not stable at 4 °C and pH 7.5. Also the concentration of benzoylecgonine in the composite samples could possibly increase by

as much as 20% over 24 h at 4 °C if cocaine was present. This would result in overestimations of cocaine use in wastewater-based epidemiology. Acidification prevents efficiently the 'formation' of benzoylecgonine from cocaine during 24-h composite sampling. For the other investigated drug residues, refrigerated sampling conditions are sufficient to prevent transformation. After sampling, drug residues need to be stable in wastewater until the actual analysis can be performed. The two most applied strategies in the literature are: 1) samples are directly frozen after collection (at −20 °C) or 2) samples are extracted using SPE cartridges within 12 h (**Table 1**). These conditions prevent degradation of the drug residues in the wastewater collected. It should be noted than when using passive samplers, analytes are extracted from the wastewater in situ, which should overcome some of these stability questions. This assumption remains to be tested however.

In view of the above mentioned data, the contribution of degradation processes occurring during in-sewer transport, sampling, and storage to the total uncertainty of the wastewater analysis methodology is expected to be negligible for several of the most commonly used illicit drugs, if the proper procedures are adopted.

Table 2. Summary of stability experiments for the main illicit drugs and several metabolites.

References	*(Castiglioni et al., 2006)*	*(Gonzalez-Marino et al., 2010)*	*(Bisceglia, 2010;Bisceglia and Lippa, 2014)*	*(Baker and Kasprzyk-Hordern, 2011)*	*(Castiglioni et al., 2011)*	*(van Nuijs et al., 2012))*	*(Plosz et al., 2013)*	*(Thai et al., 2014)*	*(Chen et al., 2013b)*	*(Senta et al 2014)*
	72 h 4 °C pH 7.5	24 h 4 °C pH 7.5	12 h 23 °C pH 7.4	12 h 19 °C pH 7.4	24 h 4 °C pH 7.5	12 h 20 °C pH 7.5	7 h 21 °C pH 7.4	12 h 20 °C pH 7.5	24 h 20 °C pH 7.0	24 h 20 °C pH 7.5
COC	-36%	-7%	-50%	-8%	-25%	-40%	-60%	-20%	-9%	-35%
BE	+14%	+7%	+10-14%	+7%	+20%	+6%	+18%	+14%	NA	+15%
EME*	NA	NA	-40%	NA	-50%	-20%	-29%	NA	NA	NA
AMP	+5%	+0%	-15%	+47%	NA	+3%	NA	NA	NA	-5%
METH	+0%	NA	+0%	+8%	NA	+2%	NA	0%	-5%	-10%
MDMA	+1%	NA	+0%	+1%	NA	+3%	NA	0%	+1%	-10%
THC-COOH	-8%	+2%	NA	NA	NA	NA	NA	NA	NA	0%
6-MAM	-14%	NA	-15%	-42%	NA	-20%	NA	-25%	-53%	-15%

COC = cocaine; BE = benzoylecgonine; EME = ecgonine methyl ester; AMP = amphetamine; METH = methamphetamine; MDMA = 3,4-methylenedioxymethamphetamine; THC-COOH = 11-nor-9-carboxy-delta9-tetrahydrocannabinol; 6-MAM = 6-monoacethylmorphine

Estimation of population size

To compare results from different sites, it is essential to know the population size contributing to the sampled wastewater (**Figure 1**). Different methods were proposed to collect information on population size and fluctuation thereof. Due to different kinds of potential biases related to each of them, it is not recommended to rely on one particular method. Currently, the population can be estimated collecting different hydrochemical parameters such as biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen (N) and phosphorus (P) and using specific loads for these parameters (i.e. per-capita loads from domestic activity) to calculate the number of people contributing to the sampled wastewater (Andreottola et al., 1994). Recently, Been and co-authors tested the possibility to normalize population using ammonium and their method appeared to be suited to show fluctuations in the size of population over long periods of time or during major events (Been et al., 2014). Another option is to collect census data in the area investigated. A comparison among the population estimates obtained from these different methods has been performed on data collected from 19 European cities and the variability ranged between 7 and 55% (RSD) (Castiglioni et al., 2013). The reliability of estimates depended on factors that could not easily be controlled, e.g. the composition of sewage (i.e. industrial, domestic or mixed) that can influence the hydro-chemical parameters, the reliability of census data, the quality of the measured flow data and the method itself used to calculate population equivalents. Considering a mean value of population estimates calculated using the different methods described is therefore not deemed appropriate due to the large biases that could be introduced into the final calculations of drug use estimates. So far, the best option available, even if not ideal, is to compile estimates based on different methods and to choose the most reliable one using expert judgement of STPs personnel. This is the procedure adopted in several recent European monitoring campaigns (Thomas et al., 2012; Ort et al., 2014a).

An interesting option is finding specific substances that, once measured in wastewater, could indicate univocally the persons served by a STP on the base of their known consumption/excretion. Nevertheless, these substances must fulfil several requirements

such as being excreted in urine in know amounts, being detectable and stable in wastewater, and originating only from human metabolism (see Chapter 3 for further details). Several potential candidates, such as creatinine, coprostanol, caffeine, pharmaceuticals, biocides and food additives have been proposed for further investigation (Daughton, 2012), and some studies tested the viability of these substances as population biomarkers in the last two years.

Due to a relatively homogenous spatiotemporal use of certain pharmaceuticals, measuring pharmaceutical loads was suggested to estimate the number of persons contributing to the sampled wastewater (Lai et al., 2011). Unfortunately, methodological challenges related to the availability of reliable prescription data of pharmaceuticals, actual consumption (patients' compliance), excretion rates and estimation of associated uncertainties remain open. However, expanding this approach from single to multiple substances is considered very promising.

Creatinine was used as a qualitative biomarker to normalize the loads of several illicit and legal drugs and this allowed studying diurnal and between-day trends by taking into account changes in population (Brewer et al., 2012). Nevertheless, the stability of creatinine has to be carefully checked within these studies, since there is evidence that its degradability in sewer conditions can affect creatinine potential to be used as a biomarker and can introduce further biases for population size estimation (Chen et al., 2014; Thai et al., 2014).

A panel of eight substances including the already propose creatinine, cholesterol, coprostanol, cotinine, and three new compounds, cortisol, androstenedione, 5-hydroxyindoleacetic acid, have been screneed as potential population biomarkers using five different criteria. These criteria included quantification methods, affinity to particulate, stability in wastewater, constancy of inter-day excretion and correlation with census population. Cotinine and 5-hydroxyindoleacetic acid resulted the most suitable compounds (Chen et al., 2014).

The concentrations of the principle metabolites of nicotine, cotinine and trans-3'-hydroxycotinine, were found to correlate with the population in the catchment areas of

several Swiss lakes and were indicated as anthropogenic markers a few years ago (Buerge et al., 2008). These substances were recently measured in raw wastewater from 8 STPs in Italy and were tested as potential population biomarkers. They were found to have a known urinary metabolism in humans, and to be easily detectable and stable in wastewater, thus it was possible to back-calculate nicotine consumption using specific correction factors. Prevalences calculated though wastewater analyses were closely comparable with those obtained from epidemiological surveys (Castiglioni et al., 2014b). Similar results were obtained in Lisbon, where only cotinine was measured in three STPs to back-calculate nicotine consumption, and results were in line with a European Survey (Lopes et al., 2013). This suggests that the amounts of nicotine metabolites measured in wastewater reflect the number of smokers in a population. With this information and the average number of cigarettes smoked per day known from epidemiological surveys, it is therefore possible to use nicotine metabolites to estimate the population size served by a STP. Further applications are now required to confirm these preliminary results.

To reduce and quantify uncertainty of population estimates it seems reasonable to combine multiple, unbiased indicators for population size measured in wastewater. One option applying Bayesan inference was recently developed by (O'Brien et al., 2014). The results based on mulitple pharmaceuticals were validated with *de facto* population enumerated on census day through a geo-referenced analysis (in Australia both *de facto* and *de jure* population are determined on census day). Therefore, no information was needed on sales data of pharmaceuticals. This approach is able to produce accurate estimates of population size for large cities, while further research is needed to improve estimates for smaller populations. Most importantly, this approach provides a reliable estimate of the uncertainty of the population estimate, implicitly including spatiotemporal variability of indicators. This cannot be obtained in the same manner with other methods. A methodological advantage of estimating population from a parameter measured in wastewater is the fact that a potential bias of flow measurements cancels out in the back-calculation (Lai et al., 2011). This is advantegous as it usually is very difficult to assess the bias of flow measurements.

Conclusions

Concerted efforts have been made in the last years to improve the wastewater-based epidemiology approach and reduce uncertainty related to community drug use estimates. A good knowledge of the critical steps and actions for improvements were provided as reported extensively within this chapter. This was made possible through the establishment of a European collaboration network (SCORE group - http://score-cost.eu/), and the collaboration of different experts such as analytical chemists, drug toxicologists and sewer engineers. The final goal will be now to start a close collaboration with drug epidemiologists to discuss future opportunities for bringing together wastewater-based epidemiology and drug epidemiology.

References

Andreottola G, Bonomo L, Poggiali L, Zaffaroni C. A methodology for the estimation of unit nutrient and organic loads from domestic and non-domestic sources. European Water Pollution Control 1994; 4: 13-19.

Baker DR, Kasprzyk-Hordern B. Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography-mass spectrometry. J Chromatogr A 2011; 1218: 8036-59.

Banta-Green C, Field J. City-wide drug testing using municipal wastewater: A new tool for drug epidemiology. Significance 2011; 8: 70-74.

Been F, Rossi L, Ort C, Rudaz S, Delemont O, Esseiva P. Population normalization with ammonium in wastewater-based epidemiology: application to illicit drug monitoring. Environ Sci Technol 2014; 48: 8162-69.

Berset JD, Brenneisen R, Mathieu C. Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography--electrospray tandem mass spectrometry (HPLC-MS/MS). Chemosphere 2010; 81: 859-66.

Bijlsma L, Emke E, Hernandez F, de Voogt P. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. Chemosphere 2012; 89: 1399-406.

Bijlsma L, Emke E, Hernandez F, de Voogt P. Performance of the linear ion trap Orbitrap mass analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water. Anal Chim Acta 2013; 768: 102-10.

Bisceglia K. PhD Dissertation. John Hopkins University, Baltimore, 2010.

Bisceglia KJ, Lippa KA. Stability of cocaine and its metabolites in municipal wastewater - the case for using metabolite consolidation to monitor cocaine utilization. Environ Sci Pollut Res Int 2014; 21: 4453-60.

Brewer AJ, Ort C, Banta-Green CJ, Berset JD, Field JA. Normalized diurnal and between-day trends in illicit and legal drug loads that account for changes in population. Environ Sci Technol 2012; 46: 8305-14.

Buerge IJ, Kahle M, Buser HR, Muller MD, Poiger T. Nicotine derivatives in wastewater and surface waters: application as chemical markers for domestic wastewater. Environ Sci Technol 2008; 42: 6354-60.

Castiglioni S, Zuccato E, Crisci E, Chiabrando C, Fanelli R, Bagnati R. Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. Anal Chem 2006; 78: 8421-9.

Castiglioni S, Zuccato E, Chiabrando C, Fanelli R, Bagnati R. Mass spectrometric analysis of illicit drugs in wastewater and surface water. Mass Spectrom Rev 2008; 27: 378-94.

Castiglioni S, Bagnati R, Melis M, Panawennage D, Chiarelli P, Fanelli R, et al. Identification of cocaine and its metabolites in urban wastewater and comparison with the human excretion profile in urine. Water Res 2011; 45: 5141-50.

Castiglioni S, Bijlsma L, Covaci A, Emke E, Hernandez F, Reid M, et al. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers. Environ Sci Technol 2013a; 47: 1452-60.

Castiglioni S, Thomas KV, Kasprzyk-Hordern B, Vandam L, Griffith P. Testing wastewater to detect illicit drugs: state of the art, potential and research needs. Sci Total Environ. 2014a; 487: 613-20.

Castiglioni S, Senta I, Borsotti A, Davoli E, Zuccato E. A novel approach for monitoring tobacco use in local communities by wastewater analysis. Tobacco Control 2014b, doi:10.1136/tobaccocontrol-2014-051553.

Chen C, Kostakis C, Irvine RJ, Felgate PD, White JM. Evaluation of pre-analysis loss of dependent drugs in wastewater: stability and binding assessments. Drug Test Anal 2013; 5: 716-21.

Chen C, Kostakis C, Gerber JP, Tscharke BJ, Irvine RJ, White JM. Towards finding a population biomarker for wastewater epidemiology studies. Sci Total Environ 2014; 487: 621-8.

Chiaia AC, Banta-Green C, Field J. Eliminating solid phase extraction with large-volume injection LC/MS/MS: analysis of illicit and legal drugs and human urine indicators in U.S. wastewaters. Environ Sci Technol 2008; 42: 8841-8.

Daughton CG. Real-time estimation of small-area populations with human biomarkers in sewage. Sci Total Environ 2012; 414: 6-21.

Emke E, Evans S, Kasprzyk-Hordern B, de Voogt P. Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: A Dutch perspective. Sci Total Environ 2013, doi.org/10.1016/j.scitotenv.2013.11.043.

Evans SE, Kasprzyk-Hordern B. Applications of chiral chromatography coupled with mass spectrometry in the analysis of chiral pharmaceuticals in the environment. Trends in Environmental Analytical Chemistry 2014; doi: 10.1016/j.teac.2013.11.005.

Gonzalez-Marino I, Quintana JB, Rodriguez I, Cela R. Determination of drugs of abuse in water by solid-phase extraction, derivatisation and gas chromatography-ion trap-tandem mass spectrometry. J Chromatogr A 2010; 1217: 1748-60.

Gonzalez-Marino I, Quintana JB, Rodriguez I, Gonzalez-Diez M, Cela R. Screening and selective quantification of illicit drugs in wastewater by mixed-mode solid-phase extraction and quadrupole-time-of-flight liquid chromatography-mass spectrometry. Anal Chem 2012; 84: 1708-17.

Harman C, Reid M, Thomas KV. In situ calibration of a passive sampling device for selected illicit drugs and their metabolites in wastewater, and subsequent year-long assessment of community drug usage. Environ Sci Technol 2011; 45: 5676-82.

Harman C, Allan IJ, Vermeirssen EL. Calibration and use of the polar organic chemical integrative sampler--a critical review. Environ Toxicol Chem 2012; 31: 2724-38.

Ibáñez M, Sancho J, Bijlsma L, van Nuijs A, Covaci A, Hernández F. Comprehensive analytical strategies based on hybrid quadrupole time of flight mass spectrometry to identify new psychoactive substances. Trends Anal. Chem. 2014; 57: 107-117.

Kasprzyk-Hordern B. Pharmacologically active compounds in the environment and their chirality. Chem Soc Rev 2010; 39: 4466-503.

Kasprzyk-Hordern B, Baker DR. Estimation of community-wide drugs use via stereoselective profiling of sewage. Sci Total Environ 2012a; 423: 142-50.

Kasprzyk-Hordern B, Baker DR. Enantiomeric profiling of chiral drugs in wastewater and receiving waters. Environ Sci Technol 2012b; 46: 1681-91.

Lai FY, Ort C, Gartner C, Carter S, Prichard J, Kirkbride P, et al. Refining the estimation of illicit drug consumptions from wastewater analysis: co-analysis of prescription pharmaceuticals and uncertainty assessment. Water Res 2011; 45: 4437-48.

Lai FY, Bruno R, Leung HW, Thai PK, Ort C, Carter S, et al. Estimating daily and diurnal variations of illicit drug use in Hong Kong: A pilot study of using wastewater analysis in an Asian metropolitan city. Forensic Sci Int 2013; 233: 126-32.

Lopes A, Silva N, Bronze MR, Ferreira J, Morais J. Analysis of cocaine and nicotine metabolites in wastewater by liquid chromatography-tandem mass spectrometry. Cross abuse index patterns on a major community. Sci Total Environ 2014; 487: 673-80.

Mathieu C, Rieckermann J, Berset JD, Schurch S, Brenneisen R. Assessment of total uncertainty in cocaine and benzoylecgonine wastewater load measurements. Water Res 2011; 45: 6650-60.

Moore KA, Mozayani A, Fierro MF, Poklis A. Distribution of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) stereoisomers in a fatal poisoning. Forensic Sci Int 1996; 83: 111-9.

O'Brien JW, Thai PK, Eaglesham G, Ort C, Scheidegger A, Carter S, et al. A model to estimate the population contributing to the wastewater using samples collected on census day. Environ Sci Technol 2014; 48: 517-25.

Ort C, Lawrence MG, Reungoat J, Mueller JF. Sampling for PPCPs in wastewater systems: comparison of different sampling modes and optimization strategies. Environ Sci Technol 2010a; 44: 6289-96.

Ort C, Lawrence MG, Rieckermann J, Joss A. Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review. Environ Sci Technol 2010b; 44: 6024-35.

Ort C, van Nuijs ALN, Berset JD, Bijlsma L, Castiglioni S, Covaci A, et al. Spatial and temporal differences in illicit drug use in Europe quantified by sewage analysis. Addiction 2014a; 109: 1338-52.

Ort C. Quality assurance/quality control in wastewater sampling. In: Zhang C, Mueller JF, Mortimer M, editors. Quality Assurance & Quality Control of Environmental Field Samples. Future Science, London, UK, 2014b.

Ort C, Eppler JM, Scheidegger A, Rieckermann J, Kinzig M, Sorgel F. Challenges of surveying wastewater drug loads of small populations and generalizable aspects on optimizing monitoring design. Addiction 2014c; 109: 472-81.

Plosz BG, Reid MJ, Borup M, Langford KH, Thomas KV. Biotransformation kinetics and sorption of cocaine and its metabolites and the factors influencing their estimation in wastewater. Water Res 2013; 47: 2129-40.

Postigo C, Lopez de Alda M, Barcelò D. Analysis of drugs of abuse and their human metabolites in water by LC-MS2: A non-intrusive tool for drug abuse estimation at the community level. Trends in Analytical Chemistry 2008a; 27: 1053-1069.

Postigo C, de Alda MJ, Barcelo D. Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography-electrospray-tandem mass spectrometry. Anal Chem 2008b; 80: 3123-34.

Ramsey M, Thompson M. Uncertainty from sampling, in the context of fitness for purpose. Accreditation and Quality Assurance 2007; 12: 503-513.

Reid MJ, Derry L, Thomas KV. Analysis of new classes of recreational drugs in sewage: Synthetic cannabinoids and amphetamine-like substances. Drug Test Anal, 2013.

Renton RJ, Cowie JS, Oon MC. A study of the precursors, intermediates and reaction by-products in the synthesis of 3,4-methylenedioxymethylamphetamine and its application to forensic drug analysis. Forensic Sci Int 1993; 60: 189-202.

Rieckermann J. On the occurrence and fate of illicit substances in sewer systems In: Frost N, Griffiths P, editors. Assessing illicit drugs in wastewater. Potential and limitations of a new monitoring approach. EMCDDA Insight 9, 2008.

Senta I, Krizman I, Ahel M, Terzic S. Assessment of stability of drug biomarkers in municipal wastewater as a factor influencing the estimation of drug consumption using sewage epidemiology. Sci Total Environ 2014; 487: 659-65.

Thai PK, O'Brien J, Jiang G, Gernjak W, Yuan Z, Eaglesham G, et al. Degradability of creatinine under sewer conditions affects its potential to be used as biomarker in sewage epidemiology. Water Res 2014; 55C: 272-279.

Thomas KV, Bijlsma L, Castiglioni S, Covaci A, Emke E, Grabic R, et al. Comparing illicit drug use in 19 European cities through sewage analysis. Sci Total Environ 2012; 432: 432-9.

Thompson M. Sampling: the uncertainty that dares not speak its name. Journal of Environmental Monitoring 1999; 1: 19N-21N.

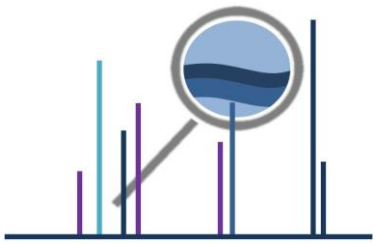
United Nation Industrial Development Organization. Complying with ISO 17025. A practical guidebook for meeting the requirements of laboratory accreditation schemes based on ISO 17025: 2005 or equivalent national standards' Vienna, 2006.

van Nuijs AL, Castiglioni S, Tarcomnicu I, Postigo C, Lopez de Alda M, Neels H, et al. Illicit drug consumption estimations derived from wastewater analysis: a critical review. Sci Total Environ 2011; 409: 3564-77.

van Nuijs AL, Abdellati K, Bervoets L, Blust R, Jorens PG, Neels H, et al. The stability of illicit drugs and metabolites in wastewater, an important issue for sewage epidemiology? J Hazard Mater 2012; 239-240: 19-23.

van Nuijs AL, Gheorghe A, Jorens PG, Maudens K, Neels H, Covaci A. Optimization, validation, and the application of liquid chromatography-tandem mass spectrometry for the analysis of new drugs of abuse in wastewater. Drug Test Anal, 2013; 6: 861-7.

Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R. Estimating community drug abuse by wastewater analysis. Environ Health Perspect 2008; 116: 1027-32.



CHAPTER 7

References

Baker DR, Kasprzyk-Hordern B.

Critical evaluation of methodology commonly used in sample collection, storage and preparation for the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by solid phase extraction and liquid chromatography-mass spectrometry.

J. Chromatogr. A *1218*, **2011**, 8036 - 8059

Baker DR, Očenášková V, Kvicalova M, Kasprzyk-Hordern B.

Drugs of abuse in wastewater and suspended particulate matter – further developments in sewage epidemiology.

Environ. Int. *48*, **2012**, 28 – 38

Baker DR, Kasprzyk-Hordern B

Spatial and temporal occurrence of pharmaceuticals and illicit drugs in the aqueous environment and during wastewater treatment: new developments.

Sci. Total Environ. *454-455*, **2013**, 442 - 456

Bagnati R, Davoli E

Analytical methods for the detection of illicit drugs in wastewaters and surface waters. In Castiglioni S, Zuccato E, (Eds.) Illicit Drugs in the Environment: Occurrence, analysis, and fate using mass spectrometry.

Wiley, Hoboken NJ (USA), ISBN: 978-0-470-52954-6 **2011**, 55 - 67

Banta-Green C, Field JA, Chiaia AC, Sudakin DL, Power L, de Montigny L

The spatial epidemiology of cocaine, methamphetamine and 3,4-methylenedioxy-methamphetamine (MDMA) use: a demonstration using a population measure of community drug load derived from municipal sewage.

Addiction. *104*, **2009**, 1874 - 1880

Baselt RC

Disposition of toxic drugs and chemicals in man (7th edition).

Biomedical publications, Foster City CA (USA), **2004**

Bartelt-Hunt SL, Snow DD, Damon T, Shockley J, Hoagland K

The occurrence of illicit drugs and therapeutic pharmaceuticals in wastewater effluent and surface waters in Nebraska.

Environ. Pollut. *157*, **2009**, 786 - 791

Berset JD, Brenneisen R, Mathieu C

Analysis of llicit and illicit drugs in waste, surface and lake water samples using large volume direct injection high performance liquid chromatography – Electrospray tandem mass spectrometry (HPLC-MS/MS).

Chemosphere *81*, **2010**, 859 - 866

Bijlsma L, Sancho JV, Pitarch E, Ibáñez M, Hernández F

Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater.

J. Chromatogr. A *1216*, **2009**, 3078 - 3089

Bijlsma L, Sancho JV, Hernández F, Niessen WMA

Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MS^E accurate-mass spectra.

J. Mass Spectrom. *46*, **2011**, 865 - 875

Bijlsma L, Emke E, Hernández F, de Voogt P

Performance of the linear ion trap Orbitrap mass analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water.

Anal. Chim. Acta *768*, **2013**, 102 - 110

Boix C, Ibáñez M, Sancho JV, Niessen WMA, Hernández F

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

J. Mass Spectrom. *48*, **2013**, 1091 - 1100

Boleda MR, Galcerán MT, Ventura F

Trace determination of cannabinoids and opiates in wastewater and surface waters by ultra-performance liquid chromatography-tandem mass spectrometry.

J. Chromatogr. A *1175*, **2007**, 38 - 48

Boleda MR, Galcerán MT, Ventura F

Monitoring of opiates and cannabinoids and their metabolites in wastewater, surface water and finished water in Catalonia, Spain.
Water Res. *43*, **2009**, 1126 - 1136

Bones J, Thomas KV, Paul B
Using environmental analytical data to estimate levels of community consumption of illicit drugs and abused pharmaceuticals.
J. Environ. Monit. 9, **2007**, 701 - 707

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

Castiglioni S, Zuccato E, Crisci E, Chiabrando C, Fanelli R, Bagnati R.
Mass spectrometric analysis of illicit drugs in wastewater and surface water.
Mass. Spectr. Rev. *27*, **2008**, 378 - 394

Castiglioni S, Zuccato E, Fanelli R (Eds.)
Illicit Drugs in the Environment: Occurrence, analysis, and fate using mass spectrometry.
Wiley, Hoboken NJ (USA), ISBN: 978-0-470-52954-6. **2011**

Castiglioni S, Bijlsma L, Covaci A, Emke E, Hernández F, Reid M, Ort C, Thomas KV, van Nuijs ALN, de Voogt P, Zuccato E
Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage drug biomarkers.
Environ. Sci. Technol. *47*, **2013**, 1452 - 1460

Castro-Perez J, Hoyes J, Major H, Preece S
Advances in MS-based approaches for drug and metabolism studies.
Chromatographia *55*, **2002**, *59 – 63*

Chiaia AC, Banta-Green C, Field J
Eliminating solid phase extraction with large-volume injection LC/MS/MS: analysis of illicit and legal drugs and human urine indicators in US wastewaters.

Environ. Sci. Technol. *42*, **2008**, 8841 - 8848

Christian GD

Analytical spectrometry (5th edition).

Wiley, Hoboken NJ (USA), ISBN: 0-471-59761-9. **1994**

Commission Decision 2002/657/EC of 12 August implementing Council Directive 96/23/EC concerning performance of analytical methods and the interpretation of results. Off. J. Eur. Commun., **2002**, L221/8

D'Asceno G, Di Corcia A, Gentili A, Mancini R, Mastropasqua R, Nazzari M, Samperi R

Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities.

Sci. Total Environ. *302*, **2003**, 199 - 209

Dass C

Fundamentals of contemporary mass spectrometry. In Desiderio DM, Nibbering NM (Eds.) Wiley-Interscience Series on Mass Spectrometry.

Wiley, Hoboken NJ (USA), ISBN: 978-0-471-68229-5, **2007**

Daughton CG

Pharmaceuticals and personal care products in the environment: Scientific and regulatory issue, Daughton CG, Jones-Lepp T (Eds.) American Chemical Society, Washington DC, **2001**, 348 - 364

Daughton CG

Illicit drugs and the environment. In Castiglioni S, Zuccato E, (Eds.) Illicit Drugs in the Environment: Occurrence, analysis, and fate using mass spectrometry.

Wiley, Hoboken NJ (USA), ISBN: 978-0-470-52954-6 **2011**, 3 - 27

De Nijs ACM, Driesprong A, den Hollander HA, de Poorter LRM, Verweij WHJ, Vonk JA, de Zwart D

Risico´s van toxische stiffen in de Nederlandse oppervlaktewateren.

RIVM rapport 607340001, Bilthoven, the Netherlands, **2008**

De Voogt P, Emke E, Helmus R, Panteliadis P, Van Leerdam JA

Determination of illicit drugs in the watercycle by LC-orbitrap MS. In Castiglioni S, Zuccato E, (Eds.) Illicit Drugs in the Environment: Occurrence, analysis, and fate using mass spectrometry.
Wiley, Hoboken NJ (USA), ISBN: 978-0-470-52954-6 **2011**, 85 - 114

European Monitoring Centre for Drugs and Drug Addiction. Insights 9: Assessing illicit drugs in wastewater. Potential and limitations of a new monitoring apporach. Luxembourg, ISBN: 978-92-9168-317-8, **2008**

European Monitoring Centre for Drugs and Drug Addiction. The state of the drug problem in Europe, Annual report. Luxembourg, ISBN: 978-92-9168-538-7, **2012**

Fatta-Kassinos D, Meric S, Nikolaou A
Pharmaceutical residues in environmental waters and wastewater: current state of knowledge and future research.
Anal. Bioanal. Chem. *399*, **2011**, 251 - 257

Farré M, Pérez S, Kantiani L, Barceló D
Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment.
Trends Anal. Chem. *27*, **2008**, 991 - 1007

Fontanals, FN, Marcé RM, Borrull F
New material in sorptive extraction techniques for polar compounds.
J. Chromatogr. A *1152*, **2007**, 14 - 31

Gheorghe A, van Nuijs A, Pecceu B, Bervoets L, Jorgens PG, Blust R, Neels H, Covaci A
Analysis of cocaine and its principal metabolites in waste and surface water using solid-phase extraction and liquid chromatography-ion trap tandem mass spectrometry.
Anal. Bioanal. Chem. *391*, **2008**, 1309 - 1319

Gilbert JR, Balcer JL, Young SA, Markham DA, Duebelbeis DO, Lewer P
Industrial applications of liquid chromatography time-of-flight mass spectrometry. In Ferrer I, Thurman EM, (Eds.) Liquid Chromatography Time-of-Flight Mass Spectrometry.

Wiley, Hoboken NJ (USA), ISBN: 978-0-470-13797-0 **2009**, 151 - 172

González-Mariño I, Quintana JB, Rodriguez I, Cela R

Determination of drugs of abuse in water by solid-phase extraction, derivatisation and gas chromatography–ion trap-tandem mass spectrometry.

J. Chromatogr. A *1217*, **2010**, 1748 - 1760

González-Mariño I, Quintana JB, Rodriguez I, González-Diéz M, Cela R

Screening and selective quantification of illicit drugs in wastewater by mixed-mode solid-phase extraction and quandrupole-time-of-flight liquid chromatography-mass spectrometry.

Anal. Chem. *84*, **2012**, 1708 - 1717

Gosetti F, Mazzucco E, Zampieri D, Gennaro MC

Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry.

J. Chromatogr. A *1217*, **2010**, 3929 - 3937

Gracia-Lor E, Sancho JV, Serrano R, Hernández F

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

Chemosphere *87*, **2012**, 453 - 462

Grabenauer M, Krol WL, Wiley JL, Thomas BF

Analysis of synthetic cannabinoids using high-resolution mass spectrometry and mass defect filtering: implication for nontargeted screening of designer drugs.

Anal. Chem. *84*, **2012**, 5574 - 5581

Hall W, Prichard J, Kirkbride P, Bruno R, Thai PK, Gartner C Lai FY, Ort C, Mueller JF

An analysis of ethical issues in using wastewater analysis to monitor illicit drug use.

Addiction *107*, **2012**, 1767 - 1773

Hernández F, Sancho JV, Pozo OJ

Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological sample.

Anal. Bioanal. Chem. *382*, **2005a**, 934 - 946

Hernández F, Pozo OJ, Sancho JV, López FJ, Marín JM, Ibáñez M

Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC-MS^2 using triple quarupole and hybrid quadrupole time-of-flight analyzers.

Trends Anal. Chem. *24*, **2005b**, 596 – 612

Hernández F, Ibáñez M, Pozo OJ, Sancho JV

Investigating the presence of pesticide transformation products in water by liquid chromatography-mass spectrometry with different mass analyzers.

J. Mass Spectrom. *43*, **2008**, 173 - 184

Hernández F, Bijlsma L, Sancho JV, Díaz R, Ibáñez M

Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight-mass spectrometry.

Anal. Chim. Acta *684*, **2011**, 96 - 106

Hernández F, Sancho JV, Ibáñez M, Abad E, Portolés T, Mattioli L

Current use of high-resolution mass spectrometry in the environmental sciences.

Anal. Bioanal. Chem. *403*, **2012**, 1251 - 1264

Hogenboom AC, Niessen WMA, Little D, Brinkman UATh

Accurate mass determinations for the confirmation and identification of organic microcontaminants in surface water using on-line solid-phase extraction liquid chromatography electrospray orthogonal-acceleration time-of-flight mass spectrometry.

Rapid Commun. Mass Spectrom. *13*, **1999**, 125 - 133

Hogenboom AC, van Leerdam JA, de Voogt P

Accurate mass screening and identification of emerging contaminants in environmental samples by liquid chromatography–hybrid linear ion trap Orbitrap mass spectrometry.

J. Chromatogr. A *1216*, **2009**, 510 - 519

Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Cooks RG

The Orbitrap: a new mass spectrometer

J. Mass Spectrom. *40*, **2005**, 430 - 443

Huerta-Fontela M, Galcerán MT, Ventura F

Ultraperformance liquid chromatography – tandem mass spectrometry analysis of stimulatory drugs of abuse in wastewater and surface waters.

Anal. Chem. *79*, **2007**, 3821 - 3829

Huerta-Fontela M, Galceran MT, Ventura F.

Stimulatory drugs of abuse in surface waters and their removal in a conventional drinking water treatment plant.

Environ. Sci. Technol. *42*, **2008a**, 6809 - 6816

Huerta-Fontela M, Galceran MT, Martin-Alonso J, Ventura F.

Occurrence of psychoactive stimulatory drugs in wastewater in north-eastern Spain.

Sci. Total Environm. *397*, **2008b**, 31 - 40

Hug C, Ulrich N, Schulze T, Brack W, Krauss M

Identification of novel micropollutants in wastewater by a combination of suspect and nontarget screening.

Environm. Pollut. *184,* **2014**, 25 - 32

Ibáñez M, Sancho JV, Pozo OJ, Niessen W, Hernández F

Use of quadrupole time-of-flight mass spectrometry in the elucidation of unknown compounds present in environmental water.

Rapid Commun. Mass Spectrom. *19,* **2005**, 169 – 178

Ibáñez M, Sancho JV, Pozo OJ, Hernández F

Use of liquid chromatography quadrupole time-of-flight mass spectrometry in the elucidation of transformation products and metabolites of pesticides. Diazon as a case study

Anal. Bioanal. Chem. *384,* **2006**, 448 - 457

Ibáñez M, Sancho JV, McMillan D, Rao R, Hernández F

Rapid non-target screening of organic pollutants in water by ultraperformance liquid chromatography coupled to time-of-flight mass spectrometry.

Trends Anal. Chem. 27, **2008**, 481 - 489

Ibáñez M, Bijlsma L, van Nuijs ALN, Sancho JV, Haro G, Covaci A, Hernández F
Quadrupole-time-of-flight mass spectrometry screening for synthethic cannabinoids in herbal blends.
J. Mass Spectrom. *48*, **2013a**, 685 - 694

Ibáñez M, Gracia-Lor E, Bijlsma L, Morales E, Pastor L, Hernández F
Removal of emerging contaminants in sewage water subjected to advanced oxidation with ozone.
J. Hazard. Mater. *260*, **2013b**, 389 - 398

Ibáñez M, Sancho JV, Bijlsma L, van Nuijs ALN, Covaci A, Hernández F
Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances.
Trends Anal. Chem. *57*, **2014**, 107 - 117

Irvine RJ, Kostakis C, Felgate PD, Jaehne EJ, Chen C, White JM
Population drug use in Australia: a sewage analysis.
Forensic. Sci. Int. *210*, **2011**, 69 - 73

Jeanville PM, Estapé ES, Torres-Negrón de Jeanville I
The effect of liquid chromatography eluents and additives on the positive ion responses of cocaine, benzoylecgonine, and ecgonine methyl ester using electrospray ionization.
Int. J. Mass Spectrom. *227*, **2003**, 247 - 258

Jones-Lepp TL, Alvarez DA, Petty JD, Huckins JN
Polar organic chemical integrative sampling and liquid chromatography-electrospray/ion-trap mass spectrometry for assessing selected prescription and illicit drugs in treated sewage effluents.
Arch. Environ. Contam. Toxicol. *47*, **2004**, 427 - 439

Karolak S, Nefau T, Bailly E, Solgadi A, Levi Y
Estimation of illicit drugs consumption by wastewater analysis in Paris area (France).
Forens. Sci. Int. *200*, **2010**, 153 - 160

Karch SB

Drug Abuse Handbook (2nd edition). In Karch SB (Ed.) CRC Press.

Taylor & Franics Group, Boca Raton FL (USA), ISBN: 0-8493-1690-1, **2007**

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ

Multi-residue method for the determination of basic/neutral pharmaceuticals and illicit drugs in surface water by solid-phase extraction and ultra performance liquid chromatography–positive electrospray ionisation tandem mass spectrometry.

J. Chromatogr. A *1161*, **2007**, 132 - 145

Kasprzyk-Hordern B, Dinsdale RM, Guwy AJ

The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters.

Water Res. *42*, **2008**, 3498 - 3518

Kasprzyk-Hordern B, Baker DR

Enantiomeric profiling of chiral drugs in wastewater and receiving waters.

Environ. Sci. Technol. *46*, **2012**, 1681 - 1691.

Kaufmann A

Quantitative analysis of veterinary drug residues by sub 2-µm particulate high performance liquid chromatography columns and time-of-flight mass spectrometry (UPLC-TOF). In Ferrer I, Thurman EM, (Eds.) Liquid Chromatography Time-of-Flight Mass Spectrometry.

Wiley, Hoboken NJ (USA), ISBN: 978-0-470-13797-0, **2009**, 133 – 150

Krauss M, Singer H, Hollender J

LC-high resolution MS in environmental analysis: from target screening to the indentificantion of unknowns.

Anal. Bioanal. Chem. 397, **2010**, 943 - 951

Kronstrand R, Josefsson M

Quantification using LC-MS. In Polettini, A (Eds.) Applications of LC-MS in Toxicology.

Pharmacuetical Press, London (UK), ISBN: 10-0-85369-629-2 **2006**, 43 - 70

Lehotay SJ, Mastovska K, Lightfield AR, Gates RA

Multi-analyst, mulit-matrix performance of the QuEChERS approach for pesticide residues in foods and feeds using HPLC/MS/MS analysis with different calibration techniques.
J AOAC Int. *93*, **2010**, 355 - 367

Liang HR, Foltz RL, Meng M, Bennett P
Ionization enhancement in atmospheric pressure chemical ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromatography/tandem mass spectrometry.
Rapid Commun. Mass Spectrom. *17*, **2003**, 2815 - 2821

Makarov A, Denisov E, Kholomeev A, Balschun W, Lange O, Strupat K, Horning S
Performance evaluation of a hybrid linear ion trap/orbitrap mass spectrometer.
Anal. Chem. *78*, **2006**, 2113 - 2120

Maralikova B, Weinmann W.
Simultaneous determination of Δ9-tetrahydrocannabinol, 11-hydroxy-Δ9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol in human plasma by high-performance liquid chromatography/tandem mass spectrometry.
J. Mass Spectrom. *39*, **2004**, 526 - 531

Mari F, Politi L, Biggeri A, Accetta G, Trignano C, Di Padua M, Bertol E
Cocaine and heroin in waste water plants: a 1-year study in the city of Florence, Italy.
Forensic Sci. Int. *198*, **2009**, 88 - 92

Marín JM, Gracia-Lor E, Sancho JV, López FJ, Hernández F
Application of ultrahigh-pressure liquid chromatography-tandem mass spectrometry to the determination of multi-class pesticides in environmental and wastewater samples. Study of matrix effects.
J. Chromatogr. A *1216*, **2009**, 1410 - 1420

Martínez-Bueno MJ, Uclés S, Hernando MD, Fernández-Alba AR

Development of a solvent-free method for the simultaneous identification/ quantification of drugs of abuse and their metabolites in environmental water by LC-MS/MS.
Talanta *85*, **2011**, 157 - 166

Matuszewski BK, Constanzer ML, Chavez-Eng CM
Startegies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS.
Anal. Chem. *75*, **2003**, 3019 - 3030

Metcalfe C, Tindale K, Hongxia L, Rodayan A, Yargeau V
Illicit drugs in Canadian municipal wastewater and estimates of community drug use.
Environ. Poll. *158*, **2010**, 3179 - 3185

Mol HGJ, Zomer P, de Koning M
Qualitative aspects and validation of a screening method for pesticides in vegetables and fruits based on liquid chromatography coupled to full scan high resolution (Orbitrap) mass spectrometry.
Anal. Bioanal. Chem. *403*, **2012**, 2891 - 2908

Murray KK, Boyd RK, Eberlin MN, Langley GJ, Li L, Naito Y
Definitions of terms relating to mass spectrometry (IUPAC Recommendations 2013).
Pure Appl. Chem. *85*, **2013**, 1515 - 1609

Nielen MWF, van Engelen MC, Zuiderent R, Ramaker R
Screening and confirmation criteria for hormone residue analysis using liquid chromatography accurate mass time-of-flight, Fourier transform ion cyclotron resonance and orbitrap mass spectrometry techniques
Anal. Chim. Acta *586*, **2007**, 122 - 129

Niessen WMA, Manini P, Andreoli R
Matrix effects in quantitative pesticide analysis using liquid chromatography - mass spectrometry
Mass Spectrom. Rev. *25*, **2006a**, 881 - 899

Niessen WMA

Liquid Chromatography-Mass Spectrometry. In Dekker M (Ed.) Chromatographic Science Series vol. 97 (3rd Edition)

CRC press, Boca Raton FL (USA), ISBN: 978-0-8247-4082-5 **2006b**

Nurmi J, Pellinen J

Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry.

J. Chromatogr. A *1218*, **2011**, 6712 - 6719

Ort C, Lawrence MG, Rieckermann J, Joss A

Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review.

Environ. Sci. Technol. *44*, **2010**, 6024 - 6035

Ort C, van Nuijs ALN, Berset J-D, Bijlsma L, Castiglioni S, Covaci A, de Voogt P, Emke E, Fatta-Kassinos D, Griffiths P, Hernandez F, González-Mariño I, Grabic R, Kasprzyk-Hordern B, Mastroianni N, Meierjohann A, Nefau T, Östman M, Pico Y, Racamonde I, Reid M, Slobodnik J, Terzic S, Thomaidis N, Thomas KV.

Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis.

Addiction *199*, **2014**, 1338 - 1352

Pedrouzo M, Borrull F, Pocurull E, Marcé RM

Drugs of abuse and their metabolites in waster and surface waters by liquid chromatography-tandem mass spectrometry.

J. Sep. Sci. *34*, **2011**, 1091 - 1101

Peters RJB, Stolker AAM, Mol JGJ, Lommen A, Lyris E, Angelis Y, Vonaparti A, Stamou M, Georgakopoulos C, Nielen MWF

Screening in veterinary drug analysis and sports doping control based on full-scan, accurate-mass spectrometry.

Trends Anal. Chem. 29, **2010**, 1250 - 1268

Petrovic M, Gros M, Barceló D

Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry.
J. Chromatogr. A *1124*, **2006**, 68 - 81

Pinhancos R, Maass S, Ramanathan DM
High-resolution mass spectrometry method for the detection, characterization and quantitation of pharmaceuticals in water.
J. Mass Spectrom. *46*, **2011**, 1175 - 1181

Pizzolato TM, Lopez de Alda MJ, Barceló D
LC-based analysis of drugs of abuse and their metabolites in urine.
Trends Anal. Chem. *26*, **2007**, 609 - 624

Plumb RS, Johnson KA, Rainville P, Smith BW, Wilson ID, Castro-Perez J, Nicholson JK
UPLC/MS^{E}; a new approach for generating molecular fragment information for biomarker structure elucidation.
Rapid Commun. Mass Spectrom. *20*, **2006**, 1989 - 1994

Postigo C, Lopez de Alda MJ, Barceló, D
Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on - line solid - phase extraction-liquid chromatography - electrospray-tandem mass spectrometry.
Anal. Chem. *80*, **2008**, 3123 - 3134

Postigo C, López de Alda M, Barceló D.
Drugs of abuse and their metabolites in the Ebro river basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation.
Environ. Int. *36*, **2010**, 75 - 84

Pozo OJ, Sancho JV, Ibáñez M, Hernández F, Niessen WMA
Confirmation of organic micropollutants detected in environmental samples by liquid chromatography tandem mass spectrometry: achievements and pitfalls.
Trends Anal. Chem. *25*, **2006**, 1030 - 1042

Quintana JB, Rodil R, López-Mahía P, Muniategui-Lorenzo S, Prada-Rodríguez D

Investigating the chlorination of acidic pharmaceuticals and the by-product formation aided by an experimental design methodology.
Water Res. *44*, **2010**, 243 - 255

Reid MJ, Derry L, Thomas KV
Analysis of new classes of recreational drugs in sewage: Synthetic cannabinoids and amphetamine-like substances.
Drug Test. Analysis. 6, **2014**, 72-79

Richardson SD, Ternes TA
Water analysis: emerging contaminants and current issues.
Anal. Chem. *83*, **2011**, 4614 - 4648

Rodayan A, Segura PA, Yargeau V
Ozonation of wastewater: Removal and transformation products of drugs of abuse.
Sci. Total Environ. *487*, **2014**, 763 - 770

Sancho JV, Pozo OJ, Ibáñez M, Hernández F
Potential of liquid chromatography/time-of-flight mass spectrometry for the determination of pesticides and transformation products in waster
Anal Bioanal. Chem. *386*, **2006**, 987 - 997

SANCO/12495/2011 (supersedes document no. SANCO/10684/2009). Method validation and quality control procedures for pesticide residues analysis in food and feed.

Schymanski EL, Singer HP, Longrée P, Loos M, Ruff M, Stravs MA, Ripollés-Vidal C, Hollender J
Strategies to characterize polar organic contamination in wastewater: exploring the capability of high resolution mass spectrometry.
Environ. Sci. Technol. 48, **2014**, 1811 – 1818

Smyth WF
Electrospray ionisation mass spectrometric behaviour of selected drugs and their metabolites.
Anal. Chim. Acta *492*, **2003**, 1 - 16

Stahnke H, Kittlaus S, Kempe G, Alder L

Reduction of matrix effects in liquid chromatrography-electrospray ionization-mass spectrometry by dilution of the sample extracts: how much dilution is needed?.
Anal. Chem. *84*, **2012**, 1474 – 1482

Ternes TA, Kreckel P, Mueller J
Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with activated sludge.
Sci. Total Environ. *225*, **1999**, 91 - 99

Terzic S, Senta I, Ahel M
Illicit drugs in wastewater of the city of Zagreb (Croatia) – estimation of drug abuse in a transition country.
Environ. Pollut. *158*, **2010**, 2686 - 2693

Thomas KV, Bijlsma L, Castiglioni S, Covaci A, Emke E, Grabic R, Hernandez F, Karolak S, Kasprzyk-Hordern B, Lindberg RH, Lopez de Alda M, Meierjohann A, Ort C, Pico Y, Quintana JB, Reid M, Rieckermann J, Terzic S, van Nuijs ALN, de Voogt P.
Comparing illicit drug use in 19 European cities through sewage analysis.
Sci. Total Environ. *432*, **2012**, 432 - 439

Thurman EM, Ferrer I
The mass defect, isotope clusters, and accurate mass for elemental determination.
In Ferrer I, Thurman EM, (Eds.) Liquid Chromatography Time-of-Flight Mass Spectrometry.
Wiley, Hoboken NJ (USA), ISBN: 978-0-470-13797-0, **2009**, 17 - 31

Tiller PR, Yu S, Castro-Perez J, Fillgrove KL, Baillie TA
High-throughput, accurate mass liquid chromatography/tandem mass spectrometry on a quadrupole time-of-flight system as a 'first-line' approach for metabolite identification studies.
Rapid Commun. Mass Spectrom. *22*, **2008**, 1053 - 1061

United Nations Office on Drugs and Crime (UNODC), World Drug Report 2013. United Nations publication (Sales No. E.13.XI.6), ISBN: 978-92-1-148273-7, Vienna (Austria), **2013**

United Nation Industrial Development Organization (UNIDO), Complying with ISO 17025. A practical guidebook for meeting the requirementsof laboratory accreditation schemes based on ISO 17025: 2005 or equivalent *national* standards: Vienna (Austria), **2006**; http://www.labnetwork.org/en/testing-areas/chemical-lab/108-proficiency-testing#6

van der Aa M, Bijlsma L, Emke E, Dijkman E, van Nuijs ALN, van de Ven B, Hernández F, Versteegh A, de Voogt P
Risk assessment for drugs of abuse in the Dutch watercylce.
Water Res. *47*, **2013**, 1848 - 1857

van Nuijs ALN, Pecceu B, Theunis L, Dubois N, Charlier C, Jorens PG, Bervoets L, Blust R, Neels H, Covaci A
Cocaine and metabolites in waste and surface water across Belgium.
Environ. Pollut. *157*, **2009**, 123 - 129

van Nuijs ALN, Castiglioni S, Tarcomnicu I, Postigo C, Lopez de Alda M, Neels H, Zuccato E, Barceló D, Covaci A
Illicit drug consumption estimations derived from wastewater analysis: A critical review.
Sci. Total Environ. *409*, **2011a**, 3564 – 3577

van Nuijs ALN, Mougel J-F, Tarcomnicu I, Bervoets L, Blust R, Jorens P, Neels H, Covaci A
Sewage epidemiology – a real-time approach to estimate the consumption of illicit drugs in Brussels, Belgium.
Environ. Int. *37*, **2011b**, 612 – 621

van Nuijs ALN
Wastewater analysis as complementary approach for estimating illicit drug consumption in the general population.
Universiteit Antwerpen, Doctoral Thesis, Antwerp (Belgium), **2012a**

van Nuijs ALN, Abdellati K, Bervoets L, Blust R, Jorens PG, Neels H, Covaci A
The stability of illicit drugs and metabolites in wastewater, an important issue for sewage epidemiology?
J. Hazard. Mater. *239-240*, **2012b**, 19 - 23

Vazquez-Roig P, Andreu V, Blasco C, Picó Y

SPE and LC-MS/MS determination of 14 illicit drugs in surface waters from the Natural Park of L'Albufera (València, Spain).

Anal. Bioanal. Chem. *397*, **2010**, 2851 - 2864

Waters Micromass

Q-Tof premier Mass Spectrometer, Operator's guide

71500089402 / Revision B

(http://www.vtpup.cz/common/manual/PrF_kach_Waters_Q-TOF_manual_EN.pdf)

Wang K-C, Shih T-S, Cheng S-G

Use of SPE and LC/TIS/MS/MS for rapid detection and quantitation of ketamine and its metabolite, norketamine, in urine.

Forensic Sci. Int. *147*, **2005**, 81 - 88

Wang P-P, Bartlett MG

Collision-induced dissociation mass spectra of cocaine, and its metabolites and pyrolysis products.

J. Mass Spectrom. *33*, **1998**, 961 - 967

Weaver PJ, Laures A, Wolff J-C

Investigation of the advanced functionalities of a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer.

Rapid Commun. Mass Spectrom. *21*, **2007**, 2415 - 2421

Wick A, Wagner M, Ternes TA

Elucidation of the transformation pathway of the opium alkaloid codeine in biological wastewater treatment.

Environ. Sci. Technol. *45*, **2011**, 3374 - 3385

Wiessing L, Vicente J, Hickman M

Integrating wastewater analysis with conventional approaches to measuring drug use. In EMCDDA Insights 9: Assessing illicit drugs in wastewater.

Luxembourg, ISBN: 978-92-9168-317-8, **2008**, 80 - 91

Wille K, Claessens M, Rappé K, Monteyne E, Janssen CR, deBrabander HF, Vanhaecke L

Rapid quantification of pharmaceuticals and pesticides in passive samplers using ultra high performance liquid chromatography coupled to high resolution mass spectrometry.
J. Chromatogr. A *1218*, **2011**, 9162 - 9173

Wills S
Drugs of Abuse (2nd edition). PhP Pharmaceutical Press, Grayslake IL (USA), ISBN: 0-85369-582-2, **2005**

Zhang H, Zhang D, Ray K
A software filter to remove interference ions from drug metabolites in accurate mass liquid chromatography/mass spectrometric analyses.
J. Mass Spectrom. *38*, **2003**, 1110 - 1112

Zuccato E, Chiabrando C, Castiglioni S, Calamari D, Bagnati R, Schiarea S, Fanelli R
Cocaine in surface water: a new evidence-based tool to monitor community drug abuse.
Environ. Health *4*, **2005**, 14 (http://www.ehjournal.net/content/4/1/14)

Zuccato E, Castiglioni S, Bagnati R, Chiabrando C, Grassi P, Fanelli R
Illicit drugs, a novel group of environmental contaminants.
Water Res. *42*, **2008a**, 961 - 968

Zuccato E, Chiabrando C, Castiglioni S, Bagnati R, Fanelli R.
Estimating community drug abuse by wastewater analysis.
Environ. Health Perspect. *116*, **2008b**, 1027 - 1032

Hyperlinks (July 2014)

Norsk institutt for vannforskning, SCORE http://www.niva.no/SCORE

Environmental Health, Zuccato *et al.* 2005 http://www.ehjournal.net/content/4/1/14

io9, blog "we come from the future"
http://io9.com/5944501/toilet-tapping-how-science-is-using-sewers-to-monitor-your-drug-habit

International Union of Pure and Applied Chemistry (IUPAC), Gold Book
http://goldbook.iupac.org/M03759.html

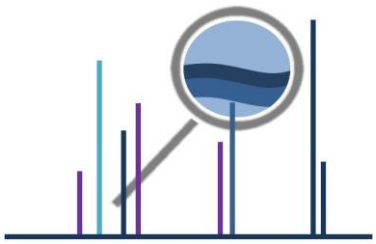
Lamond laboratory, eletrospray
http://www.lamondlab.com/MSResource/LCMS/MassSpectrometry/electrosprayIonisation.php

Rijksinstituut voor Volksgezondheid and Milieu (RIVM)
http://www.rivm.nl/dsresource?objectid=rivmp:26501&type=org&disposition=inline&ns_nc=1

United Nation Industrial Development Organization (UNIDO)
http://www.labnetwork.org/en/testing-areas/chemical-lab/108-proficiency-testing#6

Waters, Stepwave
http://www.waters.com/waters/en_US/StepWave/nav.htm?cid=134673601

Waters Micromass QTOF premier Mass Spectrometer, operator's guide
http://www.vtpup.cz/common/manual/PrF_kach_Waters_Q-TOF_manual_EN.pdf



Chapter 8

Conclusions

(English – Español - Nederlands)

Conclusions

The general conclusion of this doctoral thesis is that advanced analytical techniques, based on chromatography hyphenated to mass spectrometry, are essential to obtain accurate concentration data on excretion products of illicit drugs in water. The complementary use of low and high resolution mass spectrometry, specifically tandem mass spectrometry (MS/MS) with triple quadrupole analyzer and quadrupole-time of flight mass spectrometry (QTOF MS), allows the comprehensive investigation of illicit drugs (IDs) in the aquatic environment. This is an illustrative example where modern analytical chemistry is fundamental to advance the knowledge of use and trends of IDs in a population, and to understand the potential impact of these emerging contaminants on the aquatic ecosystem.

As a result of the research performed in this thesis, several specific conclusions can be extracted:

1. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with triple quadrupole mass analyzer is a powerful analytical technique for the quantification of IDs and their metabolites in different types of water. The robustness, sensitivity and selectivity of this technique make it highly appropriate for the determination of these compounds at the low concentrations commonly found in complex-matrix samples such as urban wastewater and surface water.
2. The application of solid-phase extraction (SPE), for sample clean-up and pre-concentration, allows reaching the sensitivity required to detect the excretion products of IDs at sub-ppb levels in urban wastewater and surface water samples.
3. Sample dilution, reduction of injection volume and the use of isotope-labelled internal standards (ILIS), have been strategies used to minimize and correct for matrix effects commonly found in LC-MS analysis.
4. The use of the analyte isotope-labelled compound as internal standard has allowed the satisfactory matrix effect correction in all the samples tested. When the analyte ILIS is not available, the use of another compound (e.g. analogue isotope-labelled internal standard) may be an adequate alternative, although the

satisfactory correction for all the samples analyzed cannot be ensured. Hence, the use of analogues ILIS should be carefully checked in a notable number of samples.

5. The development of LC-MS/MS multi-residue methods requires a compromise in the selection of experimental conditions: chromatographic separation, MS/MS detection and sample treatment. By loading 25 - 100 mL water sample onto SPE Oasis HLB cartridges (Oasis MCX have also shown satisfactory performance) it is feasible to determine 11 IDs with LOQs between 2 and 100 ng/L with satisfactory accuracy (recoveries 70 – 120%) and precision (RSD < 20%), using C_{18} UHPLC column (1.7µm, 50 mm x 2.1 mm (i.d.)) with chromatographic run times of 6 minutes.

6. The simultaneous quantification and confirmation of widely consumed IDs and their metabolites have been possible by acquiring three MS/MS transitions for each compound. Although two transitions, together with the compliance of the retention time and ion ratio is the minimum required for a safe confirmation, the acquisition of three transitions facilitates the determination of the analytes and minimizes the possibilities of reporting false negatives *i.e.* in those cases when one of the transitions is interfered and the ion ratio does not coincide with that of the reference standards, the other transition may be used.

7. The analysis of quality control samples with each sample batch, and regular participation in inter-laboratory quality exercises are essential, as it gives relevant information on the performance and robustness of the methodology applied.

8. Ultra-high-performance liquid chromatography (UHPLC) allows the development of rapid analytical methods, providing improved chromatographic resolution and increased method sensitivity. To take real profit of the benefits offered by UHPLC, fast-acquisition mass spectrometers are required able to acquire sufficient number of data points per peak.

9. UHPLC coupled to QTOF MS running in MS^E mode, has shown to be a powerful analytical tool for rapid screening (detection and identification) of IDs, prescription drugs with potential for abuse and metabolites in wastewater samples. Cocaine, benzoylecgonine (BE), codeine and cotinine were most frequently detected in

influent wastewaters, whereas codeine, BE and oxazepam were frequently found in effluents. Accurate-mass full-spectrum mass data provided by QTOF MS allow the tentative identification of compounds detected even without using reference standards. However, for unequivocal confirmation reference standards are required as a final step of the confirmatory process.

10. The combination of LC to QTOF MS or LTQ Orbitrap MS, both with high resolving power and mass accuracy, has demonstrated excellent qualitative capabilities. LTQ Orbitrap MS also showed good quantitative capabilities for the determination of IDs in complex wastewater samples, a new trend thanks to improvements of HRMS instruments in terms of sensitivity, selectivity and wider dynamic range.

11. The retrospective data evaluation in HRMS allows the identification of any compound of interest at any time without the need for additional analysis, provided that sample treatment conditions, chromatographic separation and ionization mode are suitable for the compounds to be investigated. In this work, retrospective analysis has allowed the detection of two metabolites of ketamine *i.e.* norketamine and dehydronorketamine, in water samples previously analyzed by LC-LTQ Orbitrap MS.

12. Accurate-mass data provided by LC-QTOF MS have allowed the proposal of fragmentation pathways for IDs and their metabolites. This is valuable for the selection of specific fragment ions when developing LC-MS-based analytical methods. The selection of specific product ions will minimize potential matrix interferences and will be of help to confirm the identity of the compounds detected in complex-matrix samples.

13. Accurate-mass data obtained by QTOF MS under MS^E mode and the knowledge of the fragmentation pathways of cocaine and BE has allowed the elucidation of the structures of several TPs formed in laboratory-controlled degradation experiments.

14. Cocaine and its main metabolite BE presented the highest concentrations in wastewaters of Spain (province of Castellón) and the Netherlands. Amphetamine and MDMA were frequently detected in influents from the Netherlands. However, in Spain, these designer drugs were occasionally detected in weekends, during

holiday periods and/or during an international music event. The latter also highlighted the presence of high levels of MDA.

15. Removal of IDs was estimated from the analysis of influent and effluent wastewaters of several WWTPs (using conventional primary and secondary treatment) from Spain and the Netherlands, and it was generally satisfactory and comparable between different WWTPs. Removal efficiencies of amphetamine, cocaine and its metabolites and THC-COOH were usually higher than 75%, but MDMA presented high variability with normally poor elimination. Opiates, studied only in the Netherlands, also showed high differences and lower removal efficiencies. Benzodiazepines were commonly found at higher concentrations in effluents than in influents. A significant decrease in the efficiency of the WWTP of Benicassim, Spain was observed when concentrations of IDs were high *i.e.* samples taken during a pop/rock festival.

16. It has been observed that Dutch wastewater effluents contribute to the total load of IDs and metabolites in the rivers Rhine and Meuse (the Netherlands). However, finished drinking water, produced from those rivers, did not contain any of the selected IDs, with the only exception of one water sample where BE was detected, although at a concentration below the limit of quantification. Our results suggest that no short-term toxicological relevance for environment and human health can be expected from the presence of IDs in the aquatic ecosystem. However, further research is recommended with respect to possible long-term effects on organisms and effects of combined exposure to multiple compounds.

17. Sewage-based epidemiology is a multidisciplinary approach, where analytical chemistry plays a key role. Collaboration with experts and researchers from the same and different fields helps identifying and filling gaps in existing knowledge in order to improve the robustness and applicability of the approach.

Conclusiones

La conclusión general de esta tesis doctoral es que las técnicas analíticas avanzadas, basadas en el acoplamiento cromatografía-espectrometría de masas, son esenciales para obtener datos exactos de concentración sobre los productos de excreción de drogas ilícitas en agua. El uso complementario de la espectrometría de masas de baja y alta resolución, específicamente la espectrometría en tándem (MS/MS) con analizadores de triple cuadrupolo y cuadrupolo-tiempo de vuelo (QTOF MS), permite la investigación exhaustiva de drogas ilícitas (IDs) en el medio acuático. Esto es un ejemplo ilustrativo donde la química analítica moderna es fundamental para avanzar en el conocimiento del uso y tendencias de las IDs en una población, y para entender el impacto potencial de estos contaminantes emergentes en el ecosistema acuático.

Como resultado de la investigación llevada a cabo en esta tesis, se pueden extraer varias conclusiones específicas:

1. La cromatografía líquida acoplada a espectrometría de masas en tándem (LC-MS/MS) con analizador de triple cuadrupolo es una técnica analítica poderosa para la cuantificación de IDs y sus metabolitos en diferentes tipos de aguas. La robustez, sensibilidad y selectividad de esta técnica la hace apropiada para la determinación de estos compuestos a los bajos niveles de concentración comúnmente encontrados en muestras de matriz compleja tales como aguas residuales urbanas y aguas superficiales.
2. La aplicación de la extracción en fase sólida (SPE), para la purificación y preconcentración de muestras, permite alcanzar la sensibilidad requerida para detectar los productos de excreción de IDs a niveles de sub-ppb en muestras de agua residual urbana y superficial.
3. La dilución de la muestra, la reducción del volumen de inyección y el uso de patrones internos marcados isotópicamente (ILIS), han sido estrategias utilizadas para minimizar y corregir el efecto matriz comúnmente encontrado en el análisis LC-MS.

4. El uso del analito marcado isotópicamente como patrón interno ha permitido corregir satisfactoriamente el efecto matriz en todas las muestras estudiadas. Cuando el analito ILIS no está disponible, el uso de otro compuesto (*p.e.* un patrón interno análogo marcado isotópicamente) podría ser una alternativa adecuada, aunque no se aseguraría la corrección satisfactoria de todas las muestras analizadas. Por tanto, el uso de análogos ILIS se debería controlar cuidadosamente en un notable número de muestras.

5. El desarrollo de métodos multiresiduo LC-MS/MS requiere un compromiso en la selección de las condiciones experimentales: separación cromatográfica, detección MS/MS y tratamiento de muestra. La carga de 25 - 100 mL de muestra de agua en los cartuchos SPE Oasis HLB (Oasis MCX también ha mostrado resultados satisfactorios) es fiable para determinar 11 IDs con LOQs entre 2 y 100 ng/L con exactitud satisfactoria (recuperaciones 70 – 120 %) y precisión (RSD < 20%), utilizando una columna UHPLC C_{18} (1.7µm, 50 mm x 2.1 mm (i.d.)) con tiempo de análisis cromatográfico de 6 minutes.

6. La cuantificación y confirmación simultánea de las IDs ampliamente consumidas y sus metabolitos ha sido posible con la adquisición de tres transiciones MS/MS para cada compuesto. Aunque dos transiciones, junto con el cumplimiento del tiempo de retención y la relación de iones es el mínimo requerido para una confirmación segura, la adquisición de tres transiciones facilita la determinación de los analitos y minimiza las posibilidades de reportar falsos negativos; *p.e.* en aquellos casos en los que una de las transiciones está interferida y la relación de iones no coincide con la del patrón de referencia, se podría utilizar la otra transición.

7. El análisis de las muestras de control de calidad en cada secuencia de muestras, y la participación frequente en ejercicios interlaboratorio son esenciales puesto que proporciona una información relevante en el desarrollo y la robustez de la metodología aplicada.

8. La cromatografía líquida de ultra alta resolución (UHPLC) permite el desarrollo de métodos analíticos rápidos, aportando una mejor resolución cromatográfica y un aumento de la sensibilidad del método. Para aprovechar los beneficios aportados

por la UHPLC, se requieren espectrómetros de masas de rápida adquisición para obtener suficiente número de puntos de datos por pico.

9. UHPLC acoplada a QTOF MS operando en modo MS^E, ha mostrado ser una herramienta analítica poderosa para el *screening* rápido (detección e identificación) de IDs, fármacos con potencial de abuso y metabolitos en muestras de agua residual. Cocaína, benzoilecgonina (BE), codeína y cotinina fueron las más frecuentemente detectadas en aguas residuales de entrada, mientras que codeína, BE y oxazepam fueron las más encontradas en salida. Los datos obtenidos del espectro de masas *full scan* por QTOF MS permitieron la identificación tentativa de compuestos detectados incluso sin utilizar patrones de referencia. Sin embargo, para una confirmación inequívoca se requirien patrones de referencia como paso final del proceso de confirmación.

10. La combinación de LC con QTOF MS o LTQ Orbitrap MS, ambos con alto poder de resolución y masa exacta medida ha demostrado unas excelentes capacidades cualitativas. LTQ Orbitrap MS también mostró buenas capacidades cuantitativas para la determinación de IDs en muestras complejas de aguas residuales, gracias a las mejoras introducidas en la instrumentación HRMS en cuanto a sensibilidad, selectividad y mayor rango dinámico.

11. La evaluación de datos retrospectivos en HRMS permite la identificación del compuesto de interés en cualquier momento sin necesidad de un análisis adicional, siempre y cuando las condiciones de tratamiento de muestra, separación cromatográfica y modo de ionización sean adecuadas para los compuestos investigados. En este trabajo, el análisis retrospectivo ha permitido la detección de dos metabolitos de la ketamina *p.e.* norketamina y dehidronorketamina, en muestras de agua previamente analizadas por LC-LTQ Orbitrap MS.

12. Los datos de masa exacta obtenidos por LC-QTOF MS han permitido proponer vías de fragmentación para IDs y sus metabolitos. Esto resulta valioso para la selección de iones fragmento específicos durante el desarrollo de métodos analíticos basados en LC-MS. La selección de iones producto específicos minimizará las

interferencias de la matriz y facilitará la identidad de los compuestos detectados en muestras de matriz compleja.

13. Los datos de masas exacta obtenidos por QTOF MS en modo MS^E y el conocimiento de las vías de fragmentación de la cocaína y BE ha permitido la elucidación de las estructuras de varios TPs formados en los ensayos de degradación en el laboratorio bajo condiciones controladas.
14. La cocaína y su principal metabolito BE presentaron las concentraciones más altas en las aguas residuales de España (provincia de Castellón) y los Países Bajos. Anfetamina y MDMA fueron frecuentemente detectadas en entradas de los Países Bajos. Sin embargo, estas drogas de diseño fueron ocasionalmente detectadas en los fines de semana, durante periodos de vacaciones y/o durante un evento internacional musical. En este último también resaltó la presencia de altos niveles de MDA.
15. La eliminación de IDs fue estimada a partir de los análisis de aguas residuales de entrada y salida de varias WWTPs (usando tratamiento convencional primario y secundario) de España y los Países Bajos, siendo los resultados generalmente satisfactorios y comparables entre las diferentes WWTPs. La eficacia de la eliminación de la anfetamina, cocaína y sus metabolitos y THC-COOH fue generalmente mayor del 75%, pero MDMA presentó la mayor variabilidad con una eliminación generalmente baja. Los opiáceos, únicamente estudiados en los Países Bajos, también mostraron eficacias con altas diferencias y bajos factores de eliminación. Las benzodiacepinas fueron comúnmente encontradas en más altas concentraciones en salidas que en entradas. Un descenso significativo en la eficacia de la WWTP de Benicassim, España, fue observada cuando las concentraciones de IDs fueron altas *p.e.* muestras tomadas durante un festival de pop/rock.
16. Se ha observado que las aguas residuales de las salidas de los Países Bajos contribuyen a la carga total de IDs y metabolitos en los ríos Rhine y Meuse (Países Bajos). Sin embargo, el agua potable obtenida de esos ríos no contenía ninguna de las IDs seleccionadas, con la única excepción de una muestra de agua en la cual se detectó BE, aunque a una concentración por debajo del límite de

cuantificación. Nuestros resultados sugieren que no puede esperarse ninguna relevancia toxicológica a corto plazo para el medio ambiente y la salud humana derivada de la presencia de IDs en el ecosistema acuático. Sin embargo, se recomiendan futuras investigaciones con respecto a posibles efectos a largo plazo en organismos y efectos de exposición combinada a múltiples compuestos.

17. La epidemiología basada en el análisis de aguas residuales es una aproximación multidisciplinar donde la química analítica juega un papel clave. La colaboración con expertos e investigadores del mismo y de diferentes campos ayuda a la identificación y mejora del conocimiento existente para aumentar la robustez y la aplicación de la metodología.

Conclusies

Dit proefschrift toont aan dat geavanceerde analytische technieken, gebaseerd op vloeistofchromatografie gekoppeld aan massaspectrometrie, essentieel zijn voor het nauwkeurig kwantitatief bepalen van drugs en metabolieten in water. Het complementair gebruik van lage en hoge resolutie massaspectrometrie, specifiek tandem massaspectrometrie (MS/MS) met triple quadrupool massa analysator en quadrupool time-of-flight massaspectrometrie(QTOF MS), maakt een veelomvattend onderzoek naar het voorkomen van drugs in het waterig milieu mogelijk. Dit is een illustratief voorbeeld waarbij moderne analytisch chemie een sleutelrol speelt in het vergaren van meer kennis met betrekking tot de trends en het gebruik van drugs binnen een gemeenschap, en het beter begrijpen van de potentiële impact van deze "emerging contaminants" op het water ecosysteem.

Aan het onderzoek uitgevoerd in dit proefschrift kunnen verscheidene conclusies worden ontleend:

1. Vloeistofchromatografie gekoppeld aan tandem massaspectrometrie (LC-MS/MS) met triple quadrupool massa analysator is een krachtige analytische techniek voor de kwantificering van drugs en hun metabolieten in verschillende typen water. De robuustheid, gevoeligheid en selectiviteit van deze techniek maakt het mogelijk deze stoffen in zeer lage concentraties te bepalen in monsters met een complexe matrix zoals rioolwater en oppervlaktewater.
2. De toepassing van vastefase-extractie (SPE), voor de preconcentratie en voorzuivering (clean-up) van het monster levert de vereiste gevoeligheid om drugs en metabolieten te detecteren op sub-ppb niveau in riool- en oppervlaktewater.
3. De toegepaste strategieën (i) monster verdunning, (ii) reductie van het injectie volume en (iii) het gebruik van isotoop gelabelde interne standaarden, zijn geschikt om matrix-effecten te minimaliseren en/of te corrigeren.
4. Het gebruik van de isotoop gelabelde analiet als interne standaard is effectief in het corrigeren van matrix-effecten in alle geteste monsters. Bij afwezigheid van de isotoop-analiet kan een andere stof (bijvoorbeeld analoog isotoop gelabelde

interne standaarden) een alternatief zijn. Echter, een bevredigende correctie voor alle typen geanalyseerde monsters is niet gegarandeerd, zodat het gebruik van analoge interne standaarden eerst zorgvuldig getest moeten worden in een geschikt aantal monsters van het te analyseren type water.

5. De ontwikkeling van LC-MS/MS multi-residu methoden vereist in de meeste gevallen een compromis in de selectie van experimentele condities: (i) chromatografische scheiding, (ii) MS/MS detectie en (iii) monster voorbewerking. Zo wordt voor het bepalen van 11 drugs met LOQs tussen 2 en 100 ng/L met goede nauwkeurigheid (recoveries 70 – 120%) en juistheid (RSD < 20%), 25 – 100 mL watermonster concentreerd op een SPE Oasis HLB cartridge (Oasis MCX heeft ook goede prestaties getoond), en wordt een C_{18} UHPLC kolom (1.7µm, 50 mm x 2.1 mm (i.d.)) gebruikt voor een goede chromatografische scheiding met een run tijd van 6 minuten.

6. De gelijktijdige kwantificering en bevestiging van veel gebruikte drugs en hun metabolieten is mogelijk door het verwerven van drie MS/MS transities voor iedere analiet. Hoewel twee transities, samen met de retentietijd en intensiteitverhouding van de ionen het minimum vereiste is voor een positieve bevestiging, vergemakkelijkt de acquisitie van drie transities de bepaling van de analieten en minimaliseert het rapporteren van vals negatieven. Een onwenselijke situatie, die kan optreden wanneer één transitie interfereert en de intensiteitverhouding van de ionen niet overeenkomt met die van de referentie standaard. Door de acquisitie van drie transities kan in zo´n geval de andere transitie gebruikt worden ter bevestiging.

7. De analyse van kwaliteitscontrole monsters met iedere groep monsters, en de regelmatige deelname aan inter-laboratorium oefeningen zijn essentieel. Het geeft relevante informatie met betrekking tot de prestaties en robuustheid van de toegepaste methodologie.

8. Ultra-hoge-druk vloeistofchromatografie (UHPLC) faciliteert de ontwikkeling van snelle analytische methoden met verbeterde chromatografische resolutie en gevoeligheid. Om UHPLC optimaal te gebruiken zijn snelle acquisitie

massaspectrometers nodig die een voldoende aantal data punten per piek kunnen opnemen.

9. UHPLC gekoppeld aan QTOF MS opererend in MS^E mode blijkt een krachtig analytisch instrument voor de snelle screening (detectie en identificatie) van (i) drugs, (ii) voorgeschreven geneesmiddelen met potentieel voor misbruik en (iii) metabolieten in rioolwater. Cocaïne, benzoylecgonine (BE), codeïne en cotinine zijn het meest gedetecteerd in influent rioolwater, terwijl codeïne, BE en oxazepam het meest gevonden zijn in effluenten. Accurate-massa full-spectrum massa data verkregen met QTOF MS maakt de voorlopige identificatie van stoffen mogelijk, zelfs zonder gebruik te maken van referentie standaarden. Referentie standaarden zijn echter wel vereist voor onmiskenbare bevestiging, als laatste stap in het bevestigingsproces.

10. De combinatie van LC met QTOF MS of LTQ Orbitrap MS, beiden technieken met hoog massascheidendvermogen, vertonen uitstekende kwalitatieve capaciteiten. Daarnaast heeft de LTQ Orbitrap MS, voor de bepaling van drugs in complexe rioolwatermonsters, ook goede kwantitatieve capaciteiten. Deze nieuwe trend is mogelijk dankzij de verbeteringen van HRMS instrumenten qua gevoeligheid, selectiviteit en het grotere dynamische bereik.

11. De retrospectieve evaluatie van HRMS data maakt het mogelijk om iedere stof te identificeren op ieder tijdstip zonder extra instrumentele analyse. Voorwaarde is wel dat de toegepaste condities van de monstervoorbewerking, chromatografische scheiding en ionisatiemode voor de te onderzoeken stof geschikt zijn. In dit werk heeft retrospectieve analyse de detectie van twee ketamine metabolieten mogelijk gemaakt, d.w.z. norketamine en dehydronorketamine zijn gedetecteerd in, door LC-LTQ Orbitrap MS, eerder geanalyseerde water monsters.

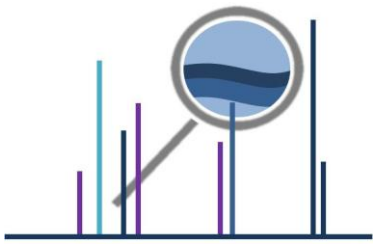
12. Data van accurate massa, verkregen met LC-QTOF MS, maakt het mogelijk om fragmentatiepatronen van drugs en hun metabolieten op te stellen. Dit is waardevol voor de selectie van specifieke fragmentionen bij de ontwikkeling van een op LC-MS gebaseerde analytische methode. De selectie van specifieke

productionen minimaliseert mogelijke matrix interferenties en bevordert de bevestiging van de identiteit van analieten in complexe matrices.

13. Accurate-massa data, verkregen met LC-QTOF MS in MS^E mode, en de kennis van de fragmentatiepatronen van cocaïne en BE faciliteert de opheldering van structuren van verscheidene TPs, gevormd tijdens laboratorium gecontroleerde degradatie experimenten.
14. De hoogste concentraties drugs in rioolwater van Spanje (de provincie Castellón) en Nederland zijn gevonden voor cocaïne en het voornaamste metaboliet ervan BE. Amfetamine en MDMA zijn frequent gedetecteerd in Nederlandse influenten. In Spanje, zijn deze designer drugs slechts in enkele gevallen gedetecteerd in weekeinden, gedurende vakantie perioden en/of gedurende een internationaal muziek evenement. Tijdens laatstgenoemd evenement zijn ook hoge concentraties van MDA aangetoond.
15. Het zuiveringsrendement van drugs door verscheidene AWZIs uit Spanje en Nederland (die gebruik maken van conventionele primaire en secundaire behandeling) kon worden geschat door de analyse van influent- en effluent-rioolwatermonsters. Het rendement was in het algemeen voldoende voor de AWZIs van beide landen. De mate van verwijdering van amfetamine, cocaïne en hun metabolieten en THC-COOH was gewoonlijk hoger dan 75%. Voor MDMA was de verwijdering onvoldoende en variabel. Opiaten, enkel bestudeerd in Nederland, gaven ook grote verschillen en meestal ook lage mate van verwijdering. De concentraties van benzodiazepinen waren vaak hoger in effluenten dan in influenten. In de AWZI van Benicassim (Spanje) werd een duidelijke afname van de verwijderingscapaciteit voor de drugs geobserveerd gedurende de week van het pop/rock festival, waarbij de concentraties van drugs in die week sterk toegenomen waren.
16. Nederlandse rioolwater effluenten dragen bij aan de totale lading van drugs in de rivieren de Rijn en de Maas. In drinkwater, geproduceerd van deze rivieren, zijn geen van de geselecteerde drugs aangetoond, met uitzondering van BE dat gedetecteerd is in slechts één monster, echter met een concentratie beneden de bepalingslimiet. Op basis van ons onderzoek kan het toxicologische risico voor het

milieu en voor volksgezondheid verwaarloosbaar worden geacht. Echter, toekomstig onderzoek aangaande de mogelijke lange termijn (chronische) effecten op organismen en mogelijke effecten van gecombineerde blootstelling aan een veelvoud van stoffen wordt aanbevolen.

17. Epidemiologie gebaseerd op rioolwater analyse (*sewage-based epidemiology*) is een multidisciplinaire benadering, waar analytische chemie een sleutelrol in heeft. Samenwerking met experts en onderzoekers van dezelfde of verschillende onderzoeksgebieden helpt het gebrek aan kennis te identificeren en in te vullen met als doel de robuustheid en toepasbaarheid van de strategie te verbeteren.



Suggestions for future research

(English – Español - Nederlands)

Suggestions for future research

This doctoral thesis has demonstrated the strong potential of LC-MS/MS, using QqQ and LC coupled to HRMS using QTOF and LTQ Orbitrap mass analyzers, for the determination of illicit drugs and related substances in complex matrices such as wastewater samples. From the results and conclusions obtained, future lines of investigation can be suggested. Part of this future research will be closely related to the SCORE network and the European funded projects of COST action (entitled: Sewage biomarker analysis for community health assessment [ref. oc-2013-1-14763]) and Marie Curie ITN, SEWPROF (entitled: A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level [ref. 317205]).

The main issues to be addressed in the future are:

- Coordinate European / worldwide comparative studies, along with inter-laboratory performance exercises.
- Contribute to a better understanding of the uncertainties associated with sewage-based epidemiology in order to improve robustness and to expand the approach to other aspects of public health such as nutrition, diseases, and lifestyle.
- Investigate the quantitative capabilities of a state-of-the-art QTOF MS (Xevo G2, Waters) for the determination of illicit drugs in wastewater samples.
- Apply the analytical strategies used in this thesis to other matrices, such as urine. This is of particular interest for monitoring urine from drug consumers, but also for toxicological studies.
- Exploiting the capabilities of LC-QTOF MS to tentatively elucidate the structures of new psychoactive substances (NPS). Approaches like searching for common fragments or mass defect filtering can be used to investigate the presence of related compounds that share a common moiety with drugs of the same chemical family.
- Contribute to gather more scientific knowledge on NPS by performing *in vitro* phase I and phase II biotransformation experiments, combined with HRMS

measurements. Subsequently, the selected *in vitro* biomarkers can be targeted in specific *in vivo* cases, such as pooled urine samples obtained from selected population (festivals, nightlife areas, etc) and wastewater from specific regions to confirm the usefulness of *in vitro* experiments. The results will be of interest for institutions like EMCDDA or UNODC, which require better knowledge, together with timely objective information regarding new shifts in the drug market and the appearance of new synthetic chemicals, in order to formulate effective drug policies.

- Widen the scope of screening by QTOF MS, including biomarkers of NPS, antidepressants, relaxants/tranquilizers, Viagra, etc (in general, pharmaceuticals with potential of abuse).
- Investigate and employ the possibilities of chromatographic retention time prediction as complementary tool for the identification of compounds, but also to reduce tedious data processing when performing a wide scope screening and when reference standards are unavailable.
- Develop analytical methodologies, based on chiral chromatography coupled with mass spectrometry, for the determination of enantiomers in wastewater. Enantiomeric profiling allows distinguishing between consumption and direct disposal of unused drugs to wastewater.

Sugerencias para futuras investigaciónes

Esta tesis doctoral ha demostrado el gran potencial de las técnicas LC-MS/MS con QqQ y LC acoplada a HRMS con analizadores QTOF y LTQ Orbitrap para la determinación de drogas ilícitas y sustancias relacionadas en matrices complejas tales como muestras de aguas residuales. De los resultados y las conclusiones obtenidas, se pueden sugerir futuras líneas de investigación. Una parte de éstas están íntimamente relacionadas con la red SCORE y los proyectos europeos de la acción COST (con título: *Sewage biomarker analysis for community health assessment* [ref. oc-2013-1-14763]) y ITN Marie Curie, SEWPROF (con título: *A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level* [ref. 317205]).

Los principales temas para un futuro son:

- Coordinación estudios comparativos europeas / mundiales, juntos con la realización de ejercicios interlaboratorio.
- Contribuir a comprender mejor las incertidumbres asociadas con la epidemiología del análisis de aguas residuales para mejorar la robustez y expandir la metodología a otros aspectos de la salud pública tales como la nutrición, las enfermedades y el estilo de vida.
- Investigar las capacidades cuantitativas del estado del arte del QTOF MS (Xevo G2, Waters) para la determinación de drogas ilícitas en muestras de agua residual.
- Aplicar las estrategias analíticas de esta tesis a otras matrices, tales como la orina. Esto resulta de particular interés para monitorizar la orina de los consumidores de drogas, así como para estudios toxicológicos.
- Explotar las capacidades del LC-QTOF MS para elucidar tentativamente las estructuras de nuevas sustancias psicoactivas (NPS). Aproximaciones tales como la búsqueda de fragmentos comunes o el filtrado de defectos de masas pueden ser utilizadas para investigar la presencia de compuestos relacionados que comparten una parte de estructura común con drogas de la misma familia química.

- Contribuir a obtener más conocimiento científico en NPS mediante ensayos *in vitro* de biotransformación fase I y fase II, en combinación con medidas por HRMS. Subsecuentemente, la selección de biomarcadores *in vitro* pueden ser medidas en modo *target* en casos específicos *in vivo*, tales como muestras mezcla de orina obtenidas de la población seleccionada (festivales, áreas de vida nocturna, etc.) y agua residual de regiones específicas para confirmar la utilidad de los ensayos *in vitro*. Los resultados serán de interés para instituciones tales como EMCDDA o UNODC, las cuales requieren de un mejor conocimiento, junto con información actual y objetiva de nuevos cambios en el mercado de las drogas y la apariencia de químicos sintéticos nuevos, con el fin de formular medidas políticas eficaces.
- Ampliar el espectro de *screening* por QTOF MS, incluyendo biomarcadores de NPS, antidepresivos, relajantes/tranquilizantes, Viagra, etc (en general, fármacos con potencial de abuso).
- Investigar y emplear las posibilidades de la predicción del tiempo de retención cromatográfico como herramienta complementaria para la identificación de compuestos, pero también para reducir el tedioso procesamiento de datos cuando se lleva a cabo un *screening* de amplio espectro y cuando los patrones de referencia no están disponibles.
- Desarrollar metodologías analíticas basadas en cromatografía quiral acoplada a espectrometría de masas para la determinación de enantiómeros en aguas residuales. El perfil enantiomérico permite distinguir entre el consumo de drogas y la eliminación directa de drogas no consumidas en las aguas residuales.

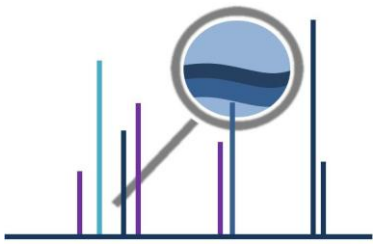
Suggesties voor toekomstig onderzoek

Dit proefschrift heeft het potentieel van LC-MS/MS, gebruikmakend van QqQ en LC gekoppeld aan HRMS met QTOF en LTQ Orbitrap massa analysatoren, voor de bepaling van drugs en gerelateerde stoffen in complexe matrices, zoals rioolwatermonsters, aangetoond. Uit de resultaten kunnen toekomstige onderzoekslijnen worden afgeleid. Een deel van dit toekomstige onderzoek zal nauw verwant zijn met het SCORE netwerk en met de door de EU gefinancierde projecten van COST actie (getiteld: Sewage biomarker analysis for community health assessment [ref. oc-2013-1-14763]) en Marie Curie ITN, SEWPROF (getiteld: A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level [ref. 317205]).

De voornaamste aandachtsgebieden voor toekomstig onderzoek zijn:

- De coördinatie van Europese / wereldwijde samenwerkingsstudies, inclusief inter-laboratorium validatieonderzoek.
- Het kwantifceren van onzekerheden, gerelateerd aan sewage-based epidemiologie voor het verbeteren van de robuustheid en de toepassing van de analytische strategie ten behoeve van uitbreiding naar andere aspecten van volksgezondheid zoals voeding, ziekten en lifestyle.
- Het onderzoeken van de kwantitatieve capaciteiten van een nieuw QTOF MS instrument (Xevo G2, Waters) voor de bepaling van drugs in rioolwatermonsters.
- Het toepassen van de analytische strategieën, ontwikkeld in dit proefschrift, op andere matrices, zoals urine. Dit is van bijzonder belang voor het meten en toezicht houden op de urine van gebruikers van drugs, en voor toxicologische studies.
- Het toepassen van LC-QTOF MS voor de structuuropheldering van nieuwe psychoactieve middelen (NPS). Strategieën zoals het zoeken naar gemeenschappelijke fragmentionen of massa defect filtering zouden kunnen worden toegepast.

- Het verkrijgen van meer wetenschappelijke kennis van NPS door het uitvoeren van *in vitro* fase 1 en fase 2 biotransformatie experimenten gecombineerd met HRMS metingen. Vervolgens kunnen de geselecteerde *in vitro* biomarkers doelgericht worden gezocht in specifieke *in vivo* situaties, zoals gemengde urine monsters verkregen van een geselecteerde populatie (festivals, uitgaansgebieden, etc) en rioolwatermonsters van specifieke gebieden om de bruikbaarheid van *in vitro* experimenten te bevestigen. Deze informatie is interessant voor instanties zoals EMCDDA of UNODC, die de verworven kennis, samen met actuele informatie over nieuwe verschuivingen en het verschijnen van nieuwe synthetische substanties op de drugs markt, gebruiken om effectief drugs beleid te kunnen formuleren.
- Het uitbreiden van de screening met QTOF MS, door biomarkers van stoffen als NPS, antidepressivum, kalmeringsmiddelen, Viagra, etc toe te voegen.
- Het voorspellen van retentietijdenals extra techniek voor de identificatie van stoffen, als ook voor de reductie van de verwerkingstijden van data die nu nodig zijn voor de uitgebreide screening zonder beschikbaarheid van referentie standaarden.
- De ontwikkeling van analytische methodologieën, gebaseerd of chirale chromatografie gekoppeld aan massaspectrometrie, voor de bepaling van enantiomeren in rioolwater. Middels enantiomeer profilering zou onderscheid gemaakt kunnen worden tussen gebruikte drugs en drugs die direct (ongebruikt) in het rioolwater worden geloosd.



Curriculum Vitae

Curriculum Vitae

Personalia

Name	Lubertus Bijlsma
Date of Birth	8 September 1977
Place of Birth	Amersfoort, the Netherlands
Nationality	Dutch

Education

1997 - 2001	BSc degree in Chemistry, Hogeschool van Utrecht, Utrecht, the Netherlands
2007 - 2008	MSc degree, Master Oficial en Técnicas Cromatográficas Aplicadas, University Jaume I, Castellón, Spain
2008 - 2014	PhD studies in Chemistry, University Jaume I, Castellón, Spain Title: The investigation of illicit drugs and their metabolites in water by liquid chromatorgaphy coupled to low and high resolution mass spectrometry (Supervisors: Prof. Dr. F. Hernández and Prof Dr. J.V. Sancho)

Employment history

2001 - 2003	Analyst A, National Institute for Public Health and the Environment (RIVM), Laboratory for Analytical Chemistry (LAC), Bilthoven, the Netherlands
2003 - 2005	Quality technician, University Jaume I, Laboratory of Pesticide Residue Analysis (LARP), Castellón, Spain
2006 - 2007	Research Analyst. St. Radboud University Nijmegen Medical Center, Clinical Pharmacy, Nijmegen, the Netherlands

Publication list

Publications and presentations (2002 – present)

Publications in peer-reviewed journals	20
Book chapters	1
Other publications	5
Oral/poster presentations at conferences	25

Total citations Web of Knowledge (July 2014): 379

Hirsch-index (July 2014): 8

Publications in peer-reviewed journals (20):

1. F. Hernández, O.J. Pozo, J.V. Sancho, **L. Bijlsma**, M. Barreda, E. Pitarch. Multiresidue LC-MS/MS determination of 52 non GC-amenable pesticides and metabolites in different food commodities. Journal of Chromatography A, 1109, (**2006**) 242 - 252 (impact factor: 4.612, ranked 6 of 75 in "Analytical chemistry"; 124 times cited).
2. **L. Bijlsma**, L.L.M. de Bruijn, E.P. Martens, M.J. Sommeijer. Water content of stingless bee honeys (Apidea, Meliponini): interspecific variation and comparison with honey of Apis mellifera. Apidologie, 37, (**2006**) 480 - 486. (impact factor: 2.155, ranked 14 of 87 in "Entomology"; 5 times cited).
3. **L. Bijlsma**, J.V. Sancho, E. Pitarch, M. Ibáñez, F. Hernández. Simultaneous ultra-high-pressure liquid chromatography-tandem mass spectrometry determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and a cannabis metabolite in surface water and urban wastewater. *Journal of Chromatography A*, 1216, (**2009**) 3078 - 3089 (impact factor: 4.612, ranked 6 of 75 in "Analytical chemistry"; 83 times cited; ***top 50 cited article*** published in Journal of Chromatography A in the period 2008-2009).

4. F. Hernández, **L. Bijlsma**, J.V. Sancho, R. Díaz, M. Ibáñez. Rapid wide-scope screening of drugs of abuse, prescription drugs with potential for abuse and their metabolites in influent and effluent urban wastewater by ultrahigh pressure liquid chromatography-quadrupole-time-of-flight mass spectrometry. Analytica Chimica Acta, 684, (**2011**) 96 - 106 (impact factor: 4.387, ranked 7 of 75 in "Analytical chemistry"; 50 times cited).

5. **L. Bijlsma**, J.V. Sancho, F. Hernández, W.M.A. Niessen. Fragmentation pathways of drugs of abuse and their metabolites based on QTOF MS/MS and MSE accurate-mass spectra. Journal of Mass Spectrometry, 46, (**2011**) 865 – 875. (impact factor: 3.214, ranked 16 of 75 in "Analytical chemistry"; 14 times cited).

6. **L. Bijlsma**, E. Emke, F. Hernández, P. De Voogt. Investigation of drugs of abuse and relevant metabolites in Dutch sewage water by liquid chromatography coupled to high resolution mass spectrometry. Chemosphere, 89, (**2012**) 1399 - 1406 (impact factor: 3.137, ranked 36 of 210 in "Environmental sciences"; 19 times cited; received the ***Eurofins Best Publication award 2012***, given by the Society of Environmental Toxicology and Chemistry (SETAC) – Europe).

7. K.V. Thomas, **L. Bijlsma**, S. Castiglioni, A. Covaci, E. Emke, R. Grabic, F. Hernández, S. Karolak, B. Kasprzyk-Horden, R.H. Lindberg, M. Lopez de Alda, A. Meierjohann, C. Ort, Y. Pico, J.B. Quintana, M. Reid, J. Rieckermann, S. Terzic, A.L.N. van Nuijs, P. De Voogt. Comparing illicit drug use in 19 European cities through sewage analysis. Science of the Total Environment, 432, (**2012**) 432 – 439 (impact factor: 3.258, ranked 31 of 210 in "Environmental sciences"; 41 times cited; ***classified as top/highly cited paper*** by ISI Web of Knowledge).

8. M. van der Aa**, L. Bijlsma**, E. Emke, E. Dijkman, A.L.N. van Nuijs, B. van de Ven, F. Hernández, A. Versteegh, P. de Voogt. Risk assessment for drugs of abuse in the Dutch watercycle. Water Research, 47, (**2013**) 1848 – 1857 (impact factor: 4.655, ranked 12 of 210 in "Environmental sciences"; 6 times cited).

9. **L. Bijlsma**, C. Boix, W.M.A. Niessen, M. Ibáñez, J. V. Sancho, F. Hernández. Investigation of degradation products of cocaine and benzoylecgonine in the aquatic environment. Science of the Total Environment, 443, (**2013**) 200 - 208

(impact factor: 3.258, ranked 31 of 210 in "Environmental sciences"; 7 times cited).

10. S. Castiglioni, **L. Bijlsma**, A. Covaci, E. Emke, F. Hernández, M. Reid, C. Ort, K.V. Thomas, A.L.N. van Nuijs, P. de Voogt, E. Zuccato. Evaluation of uncertainties associated with the determination of community drug use through the measurement of sewage biomarkers. Environmental Science and Technology, 47, (**2013**) 1452 - 1460 (impact factor: 5.257, ranked 7 of 210 in "Environmental sciences"; 21 times cited).

11. **L. Bijlsma**, E. Emke, F. Hernández, P. de Voogt. Performance of the linear ion trap Orbitrap mass analyzer for qualitative and quantitative analysis of drugs of abuse and relevant metabolites in sewage water. Analytica Chimica Acta, 768, (**2013**) 102 – 110 (impact factor: 4.387, ranked 7 of 75 in "Analytical chemistry"; 5 times cited).

12. M. Ibáñez, E. Gracia-Lor, **L. Bijlsma**, E. Morales, L. Pastor, F. Hernández. Removal of emerging contaminants in sewage water subjected to advanced oxidation with ozone. Journal of Hazardous Materials, 260, (**2013**) 389 - 398 (impact factor: 3.925, ranked 16 of 210 in "Environmental sciences"; 1 times cited).

13. M. Ibáñez, **L. Bijlsma**, A.L.N. van Nuijs, J. V. Sancho, G. Haro, A. Covaci, F. Hernández. Quadrupole-time-of-flight mass spectrometry screening for synthetic cannabinoids in herbal blends. Journal of Mass Spectrometry, 48, (**2013**) 685 - 694 (impact factor: 3.214, ranked 16 of 75 in "Analytical chemistry"; 2 times cited).

14. M. Ibáñez, J. V. Sancho, **L. Bijlsma**, A.L.N. van Nuijs, A. Covaci, F. Hernández. Comprehensive analytical strategies based on high-resolution time-of-flight mass spectrometry to identify new psychoactive substances. Trends in Analytical Chemistry, 57, (**2014**) 107 - 117 (impact factor: 6.351, ranked 2 of 75 in "Analytical chemistry").

15. C. Boix, M. Ibáñez, **L. Bijlsma**, J.V. Sancho, F. Hernández. Investigation of cannabis biomarkers and transformation products in waters by liquid chromatography coupled to time of flight and triple quadrupole mass

spectrometry. Chemosphere, 99 (**2014**), 64 – 71 (impact factor: 3.137, ranked 36 of 210 in "Environmental sciences"; 1 times cited).

16. **L. Bijlsma**, R. Serrano, C. Ferrer, I. Tormos, F. Hernández. Occurrence and behavior of illicit drugs and metabolites in sewage water from the Spanish Mediterranean coast (Valencia region). Science of the Total Environment, 487 (**2014**) 703 – 709 (impact factor: 3.258, ranked 31 of 210 in "Environmental sciences").

17. **L. Bijlsma**, E. Beltrán, C. Boix, J.V. Sancho, F. Hernández. Improvements in analytical methodology for the determination of frequently consumed illicit drugs in urban wastewater. Analytical Bioanalytical Chemistry 406 (**2014**) 4261 – 4272 (impact factor: 3.659, ranked 9 of 75 in "Analytical chemistry").

18. C. Ort, A. L.N. van Nuijs, J-D. Berset, **L. Bijlsma**, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, D. Fatta-Kassinos, P. Griffiths, F. Hernández, I. González-Mariño, R. Grabic, B. Kasprzyk-Hordern, N. Mastroianni, A. Meierjohann, T. Nefau, M. Östman, Y. Pico, I. Racamonde, M. Reid, J. Slobodnik, S. Terzic, N. Thomaidis, K. V. Thomas. Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis. Addiction, 109 (**2014**) 1338 - 1352 (impact factor: 4.746, ranked 2 of 16 in "Substance abuse").

19. C. Ort, C.J. Banta-Green, **L. Bijlsma**, S. Castiglioni, E. Emke, C. Gartner, B. Kasprzyk-Hordern, M. Reid, J. Rieckermann, A.L.N. van Nuijs. Wastewater-based epidemiology requires a truly transdisciplinary approach. GAIA (**2014**) accepted (impact factor 1.194, ranked 139 of 210 in "Environmental sciences").

20. F. Hernández, M. Ibáñez, R. Bade, **L. Bijlsma**, J.V. Sancho. Investigation of illicit drugs and pharmaceuticals in waters by liquid chromatography-high resolution mass spectrometry. Trends in Analytical Chemistry, (**2014**) under revision (impact factor: 6.351, ranked 2 of 75 in "Analytical chemistry").

Chapters in books (1):

1. F. Hernández, J.V. Sancho, **L. Bijlsma**. Wide-scope screening of illicit drugs in urban wastewater by UHPLC-QTOF MS. In: Illicit Drugs in the Environment: Occurrence, Analysis, and Fate using Mass Spectrometry, edited by S. Castiglioni, E. Zuccato and R. Fanelli (**2011**), ISBN 978-0-470-52954-6, p.69 - 85.

Other publications (5):

1. N.G.F.M. van der Aa, E. Dijkman, **L. Bijlsma**, E. Emke, B.M. van de Ven, A.L.N. van Nuijs, P. de Voogt Drugs of abuse and tranquilizers in Dutch drinking water, surface water and wastewater. (**2010**) RIVM report 703719064/2010.
2. N.G.F.M. van der Aa, E. Dijkman, B.M. van de Ven, J.F.M. van der Steegh, E. Emke, R. Helmus, P. de Voogt, **L. Bijlsma**, F. Hernández. Drugs en kalmeringsmiddelen in het oppervlaktewater [in Dutch]. H2O, (**2011**) 14/15.
3. B. Kasprzyk-Hordern, **L. Bijlsma**, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, F. Hernández, C. Ort, M. Reid, A.L.N. van Nuijs, K.V. Thomas. Wastewater profiling for drug use monitoring and community health assessment. Environment Industry Magazine (**2014**) submitted.
4. C. Ferrer, I. Tormos, F. Hernández, **L. Bijlsma**, E. Gracia-Lor, R. Serrano. Análisis de fármacos, drogas de abuso y sus metabolitos en aguas residuales de diferentes ciudades de la provincia de Castellón [in Spanish]. Tecnoaqua (**2014**) accepted.
5. S. Castiglioni, **L. Bijlsma**, A. Covaci, E. Emke, C. Harman, F. Hernández, B. Kasprzyk-Hordern, C. Ort, A.L.N. van Nuijs, P. de Voogt, E. Zuccato. Estimating community drug use through wastewater analysis. EMCDDA (**2014**) submitted.

Presentations (25):

1. G. Zomer, M.R.J. Hamzink, **L. Bijlsma**. Cholinesterase inhibitors measured with a chemiluminescence system based on GZ-11. Poster presentation at 12th International Symposium on Bioluminescence & Chemiluminescence, 2002, Cambridge, United Kingdom (co-author)
2. G. Zomer, R. Hoogerbrugge, M.R.J. Hamzink, **L. Bijlsma**. Development of an assay for antioxidant activity using acridan ester GZ-11. Poster presentation at 12th International Symposium on Bioluminescence & Chemiluminescence, 2002, Cambridge, United Kingdom (co-author)
3. G. Zomer, M.R.J. Hamzink, **L. Bijlsma**. Antioxidant capacity of various samples measured with a chemiluminescence system based on GZ-11. Poster presentation at 12th International Symposium on Bioluminescence & Chemiluminescence, 2002, Cambridge, United Kingdom (co-author)
4. **L. Bijlsma**, G. Zomer, M.J. Sommeijer. Scavenging of free radicals by honeys of different bee species. Poster presentation at Meeting of the Society for Free Radical Research, 2003, Ioannina, Greece (co-author)
5. F. Hernández, O.J. Pozo, J.V. Sancho, **L. Bijlsma**, M. Barreda, E. Pitarch. Multiresidue LC-MS/MS determination of fifty non GC-amenable pesticides in different food commodities. Poster presentation at 1st International workshop on Liquid Chromatography – Tandem Mass Spectrometry for screening and trace level quantitation in environmental and food samples, 2005, Barcelona, Spain (co-author)
6. F. Hernández, J.V. Sancho, O.J. Pozo, M. Barreda, **L. Bijlsma**, E. Pitarch, S. Grimalt Potential of LC-MS/MS for the determination of anionic, cationic and neutral pesticides and metabolites in food matrices. Poster presentation at 2nd International symposium on Recent Advances in food analyses, 2005, Prague, Czech Republic (co-author)

7. R.J.M. Brüggemann, N.M.A. Blijlevens, **L. Bijlsma**, A.C.M. Smiet, R.E. Aarnoutse, P.E. Verweij, J. Mouton, D.M. Burger, J.P. Donnelly. Pharmacokinetics of intravenous voriconazole of recipients of an allogeneic haematopoietic stem cell transplant. Poster presentation at NUCI, 2007, Nijmegen, the Netherlands (co-author)
8. **L. Bijlsma**, J.V. Sancho, E. Pitarch, F. Hernández. Simultaneous determination of amphetamine and amphetamine-like stimulants, cocaine and its metabolites, and the main metabolite of cannabis in surface waters and urban wastewaters by UPLC-MS-MS. Poster presentation at 12as Jornadas de Análisis Instrumental (JAI), 2008, Barcelona, Spain (presenter)
9. **L. Bijlsma**, J.V. Sancho, M. Ibañez, R. Díaz, E. Pitarch, F. Hernández. Rapid screening and confirmation of illicit drugs, prescriptional drugs with potential for abuse and their metabolites in biological and environmental samples by UHPLC-QTOF MS. Poster presentation at IV Reunión Sociedad Española de Espectrometría de Masas (SEEM), 2009, Castellón, Spain (presenter)
10. M. Ibañez, R. Díaz, F.J. López, J.V. Sancho, E. Gracia, **L. Bijlsma**, F. Hernández. Development and qualitative validation of a wide scope screening of emerging contaminants in natural water and wastewater by UHPLC-QTOF MS. Poster presentation at 28th International Symposium on Chromatography, 2010, Valencia, Spain (co-author)
11. M. Ibañez, C. Boix, **L. Bijlsma**, J.V. Sancho, F. Hernández. LC-QTOF MS elucidation of organic contaminants transformation products after water chlorination. Poster presentation at Symposium on emerging pollutants, water treatment and remediation, 2011, Barcelona, Spain (co-author)
12. J.V. Sancho, **L. Bijlsma**, C. Boix, F. Hernández. Potential of LC-QTOF MS for investigation of drugs of abuse in the aquatic environment. Poster presentation at 21st SETAC Europe Annual Meeting, 2011, Milan, Italy (presenter)
13. **L. Bijlsma**, E. Emke, R. Serrano, F. Hernández, W.P. de Voogt. Monitoring of drugs of abuse in Dutch sewage water by LC-LTQ FT Orbitrap MS. Poster Spotlight presentation at 21st SETAC Europe Annual Meeting, 2011, Milan, Italy (presenter)

14. J.V. Sancho, M. Ibáñez, **L. Bijlsma**, F. Hernández. Targeted and untargeted screening, quantification and confirmation of organic contaminants in waters using UPLC-QTOF MS: The positive workflow. Poster presentation at 13as Jornadas de Análisis Instrumental (JAI), 2011, Barcelona, Spain (co-author)

15. J.V. Sancho, M. Ibáñez, **L. Bijlsma**, F. Hernández. Screening of herbal blends for legal highs by UHPLC-QTOF MS. Oral presentation at Scientific meeting of the Spanish Society of chromatography and related techniques, 2012, Tarragona, Spain (co-author)

16. M. Ibáñez, E. Gracia-Lor, **L. Bijlsma**, J.V. Sancho, F. Hernández. Importance of MS selectivity and chromatographic separation in LC-MS/MS based methods when investigating pharmaceutical metabolites in water. Dipyrone as a case study. Poster presentation at 8th Annual LC/MS/MS workshop on environmental applications and food safety, 2012, Barcelona, Spain (co-author)

17. T. Portolés, J. G. J. Mol, J. V. Sancho, **L. Bijlsma**, F. Hernández. Screening of organic contaminants by GC-(Q)TOF MS using APCI and EI sources. Poster presentation at NORMAN workshop, 2012, Amsterdam, the Netherlands (presenter)

18. C. Boix, **L. Bijlsma**, M. Ibáñez, J. V. Sancho, F. Hernández. Determination of cannabis and their transformation products in water samples by UHPLC coupled to QTOF and QqQ mass analyzers. Poster presentation at NORMAN workshop, 2012, Amsterdam, the Netherlands (co-author)

19. A. Causanilles, E. Emke, **L. Bijlsma**, P. de Voogt. Analysis of drugs of abuse in sewage water by liquid chromatography coupled to high resolution mass spectrometry. Poster presentation at Netherlands Organisation for Scientific Research (NOW), 2013, Lunteren, the Netherlands (co-author)

20. **L. Bijlsma**, R. Bade, J.V. Sancho, E. Beltrán, F. Hernández. Modern analytical methodology based on UHPLC-MS/MS for the sensitive determination of illicit drugs in urban wastewaters with emphasis on amphetamine and derivatives. Poster presentation at 14th EuCheMS International Conference on Chemistry and the Environment, 2013, Barcelona, Spain (co-author)

21. **L. Bijlsma**. Investigation of drugs of abuse and relevant metabolites in the Dutch watercycle. Oral presentation at I Workshop ISIC 2012/016 Envi Food, 2013, Castellón, Spain (invited speaker)
22. **L. Bijlsma**, E. Emke, P. de Voogt. High resolution analysis: screening possibilities and potentials. Oral presentation at Testing the Water Conference, 2013, Lisbon, Portugal (invited speaker)
23. C. Ort, C. Banta-green, F. Béen, **L. Bijlsma**, S. Castiglioni, E. Emke, J. Field, C. Gartner, B. Kasprzyk-Hordern, F.Y. Lai, J. Prichard, M. Reid, M. Kinzig, A.L.N. van Nuijs. Sewage analyses as an early detection system for diseases and indicator of various public health aspects. Oral presentation at GRF Davos One Health Summit, 2013, Davos, Switzerland (co-author)
24. **L. Bijlsma**. Identification and elucidation of transformation /degradation products using high resolution mass spectrometry. Oral presentation at EU-International Training Network SEWPROF, Training Course 3: Analytical techniques for biomarkers analysis, 2014, Nieuwegein, the Netherlands (invited speaker)
25. J.V. Sancho, C. Boix, M. Ibáñez, J.R. Parsons, **L. Bijlsma**, P. de Voogt, F. Hernandez. Investigation of pharmaceuticals tranformation products in waters by UHPLC-QTOF MS. Oral presentation at 10th LC/MS/MS workshop on environmental applications and food safety, 2014, Barcelona, Spain (co-author)

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Environmental Science and Technology
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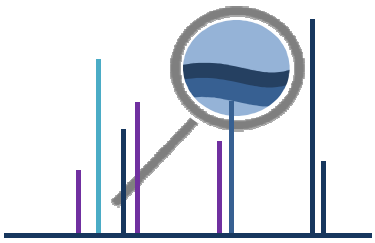

Acknowledgments / Agradecimientos / Dankwoord

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This is it. I wrote my thesis.
As a cyclist and big cycling fan, a dear friend told me once that writing your thesis is like riding a mountain stage in the "tour or vuelta". Well, I just crossed the finish line and it has been an intense but wonderful stage, in which I learned a lot, both scientifically and socially. However the work, which you hopefully have read with pleasure, wouldn´t have been written without the help and support of various important persons. It is thanks to them that I could finish my research successfully.

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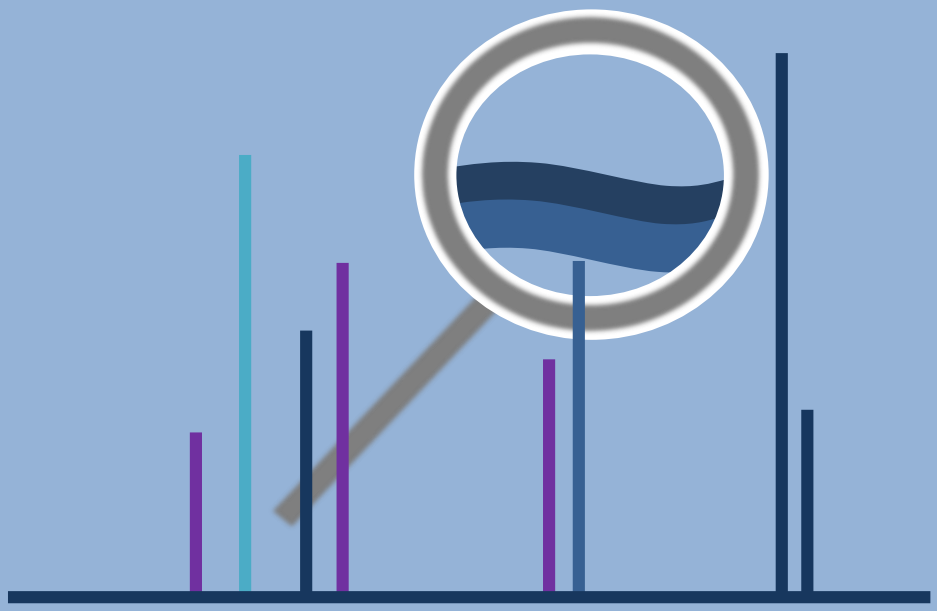

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