NEW DEVELOPMENTS TO REFINE TARGET, SUSPECT AND NON-TARGET SCREENING STRATEGIES FOR COMPREHENSIVE MONITORING OF THE AQUATIC ENVIRONMENT

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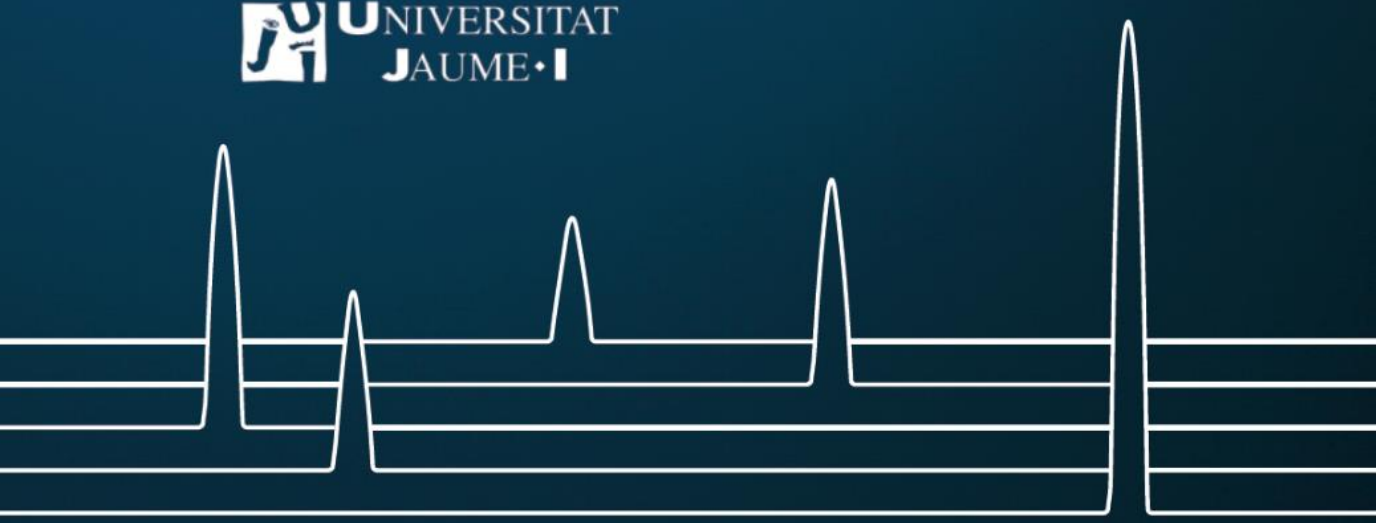

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Thesis presented by Alberto Celma Tirado in fulfilment of the requirements for the degree of Doctor (PhD) from the Universitat Jaume I

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III. "Development of a Retention Time Interpolation scale (RTi) for liquid chromatography coupled to mass spectrometry in both positive and negative ionization modes". **Alberto Celma**, Lubertus Bijlsma, Francisco López, Juan V. Sancho. *Journal of Chromatography A*, 1568 (2018), 101-107. DOI: 10.1016/j.chroma.2018.07.030. Impact factor 4.759 (2020).

IV. "Prediction of Collision Cross Section Values for Small Molecules: Application to Pesticide Residue Analysis". Lubertus Bijlsma*, Richard Bade*, **Alberto Celma**, Lauren Mullin, Gareth Cleland, Sara Stead, Félix Hernández, and Juan V. Sancho. *Analytical Chemistry*, 89 12 (2017), 6583-6589. DOI: 10.1021/acs.analchem.7b00741. (* Co-first authors) Impact factor 6.986 (2020).

Doctoral candidate contributions: formal analysis, data curation, investigation, writing – original draft, review and editing.

V. "Dual prediction of retention time and collision cross section of emerging organic contaminants for environmental analyses". **Alberto Celma***, Richard Bade*, Melissa Humphries, Juan V. Sancho, Félix Hernández and

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Doctoral candidate contributions: conceptualization, data analysis, data curation, method validation, writing – original draft, review and editing.

VI. "In vitro bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline". **Alberto Celma**, Geeta Mandava, Agneta Oskarsson, Juan Vicente Sancho, Lubertus Bijlsma, Johan Lundqvist. *Environmental Sciences Europe* 33:70 (2021) 1-12. DOI: 10.1186/s12302-021-00510-1. Impact factor 5.893 (2020).

VII. "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted. Impact factor 11.236 (2020).

VIII. "Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations". Lubertus Bijlsma*, **Alberto Celma***, Francisco López, Félix Hernández. *Current Opinion in Environmental Science & Health*, 9 (2019), 1-12. DOI: 10.1016/j.coesh.2019.03.002. (* Co-first authors). Journal of recent creation, no impact factor yet available in 2020.

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IX. "Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology". **Alberto Celma**, Juan V. Sancho, Noelia Salgueiro-González, Sara Castiglioni, Ettore Zuccato, Félix Hernández and Lubertus Bijlsma. *Journal of Chromatography A*, 1602 (2019), 300-309. DOI: 10.1016/j.chroma.2018.07.030. Impact factor 4.759 (2020).

X. "Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial". Lubertus Bijlsma, Richard Bade, Frederic Been, **Alberto Celma**, Sara Castiglioni. *Analytica Chimica Acta*, 1145 (2021), 132-147. DOI: 10.1016/j.aca.2020.08.058. Impact factor 6.558 (2020).

Doctoral candidate contributions: conceptualization, literature review, data curation, investigation, writing – original draft, review and editing.

"***This thesis has been accepted by the co-authors of the publications listed above that have waved the right to present them as a part of another PhD thesis***"

This thesis has been developed and will be defended according to the requirements of the PhD program with international mention:

i. The main language used in the present thesis is English. The summary, objectives, conclusions and suggestions for future work sections have also been included in Spanish.

ii. The candidate has performed a research stay in the Department of Biomedical Sciences and Veterinary Public Health of the Swedish University of Agricultural Sciences (Uppsala, Sweden) from 29th of March 2019 to 4th of July 2019, under the supervision of Dr. Johan Lundqvist.

iii. Prior to the defense, this work has been evaluated by three international independent reviewers directly related with the research field, Prof. Dr. Marja H. Lamoree (Vrije Universiteit Amsterdam, The Netherlands), Dr. Nikiforos Alygizakis (National and Kapodistrian University of Athens, Greece) and Dr. Sylvain Merel (French National Research Institute for Agriculture, Food and Environment, France). Additionally, two national independent reviewers also evaluated this work, Dr. Tania Portolés (University Jaume I, Spain) and Prof. Dr. Nestor Etxebarria (University of the Basque Country, Spain).

iv. The thesis will be defended in Spanish and English, and at least one international expert from a non-Spanish university will be part of the tribunal panel.

A *"l' Abuelo"* i *"l' Abuela",*

A *Ito* e *Ita*.

"*I am among those who think that science has great beauty. A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales*."

Marie Curie (1867 – 1934)

("Me encuentro entre aquellos que creen que la ciencia tiene una gran belleza. Un científico en su laboratorio no es un mero técnico: es también un niño observando fenómenos naturales que le impresionan como si de un cuento de hadas se tratara")

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Summary

Pollution of the aquatic environment has been accompanying humankind ever since the first gatherings of humans into stable settlements for a prolonged period of time. However, with the appearance of the first megacities, the increase of industrial production as well as the voracious consumerism of modern societies, waste discharges treatments are often inefficient and, therefore, the amount of organic micropollutants (OMP) being released to the aquatic environment is incommensurable. Thus, different analytical strategies have been developed over the recent years to monitor the occurrence of OMP in environmental samples so decision and policy makers have a more comprehensive snapshot of the actual situation of water bodies to promulge appropriate regulations.

In this thesis, different tools have been applied to refine target, suspect and non-target screening strategies for the monitoring of the aquatic environment with special emphasis on the hyphenation of ion mobility separation (IMS) to high resolution mass spectrometry (HRMS). This thesis has been divided into four main chapters. Briefly, the first one explores the applicability of ultra-high performance liquid chromatography (UHPLC) coupled to IMS-HRMS for the analysis of environmental samples. The second chapter deals with the development of refinement tools for suspect screening strategies such as retention time indexing or *in silico* prediction of IMS data. Then, the third part explores the application of effect-directed analysis (EDA) for a comprehensive overview of the quality of water bodies. Finally, the monitoring of a specific and challenging group of substances (new psychoactive substances, NPS) in complex matrices such as influent wastewater (IWW) and pooled urine (PU) samples is evaluated, with special aid of the previously developed tools. The studies carried out in this thesis have resulted in 10 scientific articles published in peer-reviewed journals.

The first chapter of the thesis encompasses two scientific articles devoted to explore the application of IMS-HRMS for target and suspect screening of OMP in surface water and wastewater. In brief, IMS separates ions based on their shape

and size. The time taken for an ion to travel through a mobility device can be translated into its collision cross section (CCS). This value gives an overall estimation of the size of the sphere created by the ion when moving across a gas phase. The measurement of CCS permits to introduce a new parameter for identification purposes. In **scientific article 1**, a target database for 556 OMP was developed and curated with information on retention time (RT), CCS as well as accurate mass for precursor and product ions for 970 adducts. This database is online available through the Suspect List Exchange platform from the NORMAN network as well as in Zenodo repository. Moreover, the additional value provided by CCS measurement for the identification of OMP was explored and its inclusion into widely adopted identification criteria in the environmental field was proposed in close collaboration with Dr. Emma L. Schymanski. The second work (**scientific article 2**) depicts the role, potential and main benefits gathered through the utilization of IMS-HRMS instruments for environmental screening. By putting emphasis on when and with which purpose IMS can be of help in screening strategies, several benefits are pointed out such as the reduction in the number of false positive detections and the additional separation provided by IMS which extraordinarily improves the quality of the MS data gathered. Moreover, CCS robustness measurement and isomeric resolution are discussed with illustrative examples.

The second chapter of the thesis is divided in three scientific articles. **Scientific article 3** presents the development of a RT interpolation (RTi) system in order to provide robust and valuable information when using conventional UHPLC-HRMS instruments. The approach was similar to the Kovats index. However, in this case, 16 isotopically labeled reference standards were used as RTi makers. In this sense, spiked compounds can be easily differentiated from naturally occurring ones. To prove the robustness of the developed methodology, different modifications of chromatographic separation (mobile phase composition, gradient, column chemistry, etc.) were tested. In the two following publications, *in silico* prediction of CCS values and both CCS and RT were explored as an additional tool for enhanced suspect screening strategies. In **scientific article 4**, CCS prediction model was constructed based on artificial neural networks

(ANNs) for its application in small molecule calculations. The model was developed and validated with a database of 250 molecules and achieved an accuracy for CCS prediction of ±6% for protonated molecules within 95% confidence interval. Later, in **scientific article 5**, a second model was developed by means of multiple adaptive regression splines (MARS) for the prediction of both CCS and RT values. In this case, the target database of 556 OMP previously presented was used for model development and validation. With the MARS model, the prediction accuracy improved to ± 4% for CCS of protonated molecules and ± 2 min for RT.

In the third part of this thesis, two scientific articles are presented as a result of an international research stay at the Swedish University of Agricultural Sciences (SLU) in Uppsala (Sweden) under the supervision of Dr. Johan Lundqvist, Dr. Lutz Ahrens and Prof. Karin Wiberg. In this chapter, efforts were devoted to explore EDA as a comprehensive tool to monitor quality of Spanish Mediterranean coastline water bodies. In this sense, **scientific article 6** presents the biological analysis of a set of 11 samples from relevant water bodies over a panel of 8 different bioassays. The toxicity endpoints to evaluate were selected based on their importance in environmental water quality monitoring campaigns as well as their potential to reflect toxicity due to the presence of OMP. In the subsequent study (**scientific article 7**), a wide scope target and suspect screening of OMP in the same samples was carried out by means of both UHPLC-IMS-HRMS and UHPLC coupled to low resolution mass spectrometry. Additionally, the OMPs identified in the samples were compared with observed bioactivities to integrate both strategies into a more comprehensive approach. To this purpose, online available *in silico* prediction models for toxicity were also implemented.

Finally, the last chapter of this thesis includes three scientific articles devoted to the analysis of NPS in complex matrices such as IWW and PU. The analytical strategies developed in previous chapters were also of help to overcome some of the major challenges of NPS monitoring. **Scientific article 8** presents a review of different methodologies and approaches for monitoring NPS consumption through IWW and PU analysis published within the period 2013-2018. As

highlighted in this work, IWW analysis together with PU samples collected at specific settings, *e.g.* music festival or nightlife clubs, represent a valuable source of information to give light on the type and number of different NPS consumed. In **scientific article 9**, a methodology for the quantitative determination of 22 illicit drugs and NPS in wastewater by UHPLC-MS/MS is presented. Due to the expected low concentrations of NPS in IWW, a selective and sensitive method by means of triple quadrupole mass analyzer was developed and validated. Additionally, the potential application of micro fluidic chromatography for the analysis of NPS was explored. Even though an outstanding improvement in sensitivity was observed (14-fold increase in average), the performance of micro liquid chromatography was not robust enough to permit a method validation. Finally, **scientific article 10** was completed in the form of a tutorial article presenting the main perspectives and challenges faced during the determination of NPS. The associated matrix complexity, sample preparation strategies, the high sensitivity and selectivity required and the rapid transience of NPS on the market are thoroughly discussed. Also, the pros and cons of different acquisition workflows and data exploration approaches to improve the identification capabilities in complex matrices are examined with special emphasis on IMS and *in-silico* prediction tools previously presented in this thesis.

Resumen

La contaminación del medioambiente acuático ha acompañado a la humanidad desde el primer momento en que los humanos se establecieron en asentamientos estables por un periodo prolongado de tiempo. Sin embargo, con la aparición de las primeras grandes ciudades, el incremento de la producción industrial y el voraz consumismo de las sociedades modernas, los tratamientos de los vertidos de residuos son a menudo ineficaces y, en consecuencia, la cantidad de contaminantes orgánicos (OMP) liberados al medioambiente acuático es incalculable. Así pues, en los últimos años se han desarrollado diferentes estrategias analíticas para controlar la incidencia de OMP en muestras medioambientales de tal forma que aquellos responsables políticos encargados de tomar decisiones al respecto tengan una visión más completa de la situación actual de los medios acuáticos para promulgar regulaciones adecuadas.

En esta tesis se han aplicado diferentes herramientas para perfeccionar los cribados dirigidos, de sospechosos y no dirigidos para la monitorización del medioambiente acuático con especial énfasis en el acoplamiento de la separación por movilidad iónica (IMS) con espectrometría de masas de alta resolución (HRMS). Esta tesis se ha dividido en cuatro capítulos principales. Brevemente, el primero de ellos explora la aplicabilidad de la cromatografía líquida de ultra alta resolución (UHPLC) acoplada a IMS-HRMS para el análisis de muestras ambientales. El segundo capítulo trata el desarrollo de herramientas de mejora de las estrategias de cribado como, por ejemplo, índices de tiempo de retención o predicción computacional de valores de IMS. El tercer capítulo explora la aplicación de análisis basados en efectos (EDA) para una completa visión de conjunto de la calidad de los medios acuáticos. Finalmente, en el cuarto capítulo se ha evaluado la monitorización de un grupo de sustancias particularmente complicadas (nuevas sustancias psicoactivas, NPS) en matrices complejas como el agua residual influente (IWW) o la orina (PU) con especial énfasis en las herramientas previamente desarrolladas. Los estudios realizados en esta tesis han resultado en 10 artículos científicos publicados en revistas revisadas por pares.

El primer capítulo de la tesis comprende dos artículos científicos dedicados a explorar la aplicación de IMS-HRMS para el cribado dirigido y de sospechosos de OMP en aguas superficiales y residuales. Brevemente, IMS es capaz de separar los iones en función de su forma y tamaño. El tiempo que un ion necesita para atravesar la celda de movilidad puede, posteriormente, traducirse en su sección transversal de colisión (CCS). Dicho valor proporciona una estimación global del tamaño de la esfera creada por el ion al moverse a través de un gas. La medición de valores de CCS permite introducir un nuevo parámetro de identificación. En el **artículo científico 1**, se ha desarrollado una base de datos para 556 OMPs con información acerca de tiempo de retención (RT), CCS, así como masa exacta para iones precursor y producto de un total de 970 aductos. Esta base de datos está disponible en línea a través de la plataforma 'Suspect List Exchange' de la red NORMAN así como en el repositorio virtual Zenodo. Además, se ha estudiado el valor adicional proporcionado por la medición de CCS para la identificación de OMP incorporándolo en criterios de identificación de OMP ampliamente aceptados por la comunidad científica. Esto último se ha hecho en estrecha colaboración con la Dra. Emma L. Schymanski. El segundo trabajo (**artículo científico 2**) describe el papel, potencial y principales beneficios obtenidos con la utilización de instrumentos IMS-HRMS para el análisis medioambiental con especial atención al momento y el objetivo en el que IMS puede ser de utilidad. Por ejemplo, la reducción en el nombre de detecciones de falsos positivos y la separación adicional proporcionada por IMS que mejora considerablemente la calidad de los datos de MS obtenidos. Asimismo, se discute la robustez en la medida de valores de CCS y la resolución de sustancia isómeras.

El segundo capítulo de la tesis está dividido en tres artículos científicos. El **artículo científico 3** presenta el desarrollo de un sistema de interpolación de RT (RTi) para proporcionar información útil y robusta en instrumentos UHPLC-HRMS convencionales. La estrategia seguida fue similar al índice de Kovats, aunque en este caso se ha hecho uso de 16 patrones de referencia marcados isotópicamente como marcadores de RTi. En este sentido, los compuestos fortificados en la muestra se pueden diferenciar fácilmente de los que ocurren naturalmente en la muestra. Para probar la robustez de la metodología

desarrollada se testaron diferentes modificaciones en la separación cromatográfica (composición de la fase móvil, gradiente, química de la columna, etc.). En las dos siguientes publicaciones se exploró la predicción computacional de valores de CCS o ambos CCS y RT como una herramienta adicional para la mejora de estrategias de cribado de sospechosos. En el **artículo científico 4**, se construyó un modelo de predicción de CCS basado en redes neuronales artificiales (ANNs) para su aplicación en moléculas pequeñas. El modelo fue desarrollado y validado con una base de datos de 250 moléculas consiguiendo una precisión en la predicción de ± 6% para moléculas protonadas con un intervalo de confianza del 95%. Posteriormente, en el **artículo científico 5**, se desarrolló un segundo modelo mediante *multiple adaptive regression splines* (MARS) para la predicción de RT y CCS. En este caso, la base de datos desarrollada anteriormente con información de 556 OMP fue la utilizada para el desarrollo y validación del método. Mediante el modelo MARS, la precisión de la predicción mejoró hasta el ± 4% para CCS de moléculas protonadas y ± 2 min para RT.

En la tercera parte de esta tesis se presentan dos artículos científicos como resultado de una estancia predoctoral internacional en la *Swedish University for Agricultural Sciences* (SLU) en Uppsala (Suecia) bajo la supervisión del Dr. Johan Ludqvist, Dr. Lutz Ahrens y Prof. Karin Wiberg. En este capítulo los esfuerzos se centraron en explorar EDA como una herramienta completa para la monitorización de la calidad del agua en aguas superficiales la costa Mediterránea de España. En este sentido, el **artículo científico 6** presenta el análisis biológico de 11 muestras de masas de agua relevantes frente a un panel de 8 bioensayos diferentes. Los ensayos de toxicidad a evaluar fueron seleccionados en base a su importancia en el control de la calidad ambiental de las aguas, así como por su potencial para reflejar toxicidad debida a la presencia de OMP. En el siguiente estudio (**artículo científico 7**), se llevó a cabo un cribado de amplio espectro de OMP en dichas muestras mediante UHPLC-IMS-HRMS y UHPLC acoplado a espectrometría de masas de baja resolución. Adicionalmente, los OMP identificados en las muestras se compararon con la toxicidad observada para integrar ambas estrategias en un conjunto más completo. Con este fin, se

implementó la predicción de toxicidad en línea mediante modelos computacionales.

Finalmente, el último capítulo de esta tesis incluye tres publicaciones científicas centradas en el análisis de NPS en muestras complejas como IWW y PU. Las estrategias analíticas desarrolladas en los capítulos anteriores fueron de ayuda para superar algunos de los mayores retos en el control de NPS. El **artículo científico 8** presenta una revisión de las diferentes metodologías y estrategias para la monitorización del consumo de NPS mediante el análisis de IWW y PU publicadas en el periodo 2013-2018. Tal y como se destaca en el trabajo, el análisis de IWW junto con el muestreo de PU en lugares específicos, *p. ej*. festivales de música o discotecas, representa una valiosa fuente de información para arrojar luz en el tipo y número de diferentes NPS consumidas. En el **artículo científico 9** se presenta una metodología para la determinación cuantitativa de 22 drogas ilícitas y NPS en aguas residuales mediante UHPLC-MS/MS. Debido a las esperadas bajas concentraciones de NPS en IWW, se desarrolló y validó un método sensible y selectivo mediante analizador de masas de tipo triple cuadrupolo. Además, se exploró el potencial de la cromatografía micro fluídica para el análisis de NPS. Aunque se observó un aumento extraordinario de la sensibilidad (incremento medio de un factor 14), la separación con cromatografía micro fluídica no fue suficientemente robusta para permitir la validación del método. Finalmente, el **artículo científico 10** se completó en forma de un tutorial presentando las principales perspectivas y retos encontrados durante la determinación de NPS. Se discuten en profundidad la complejidad de la matriz, las estrategias de tratamiento de muestra, la elevada sensibilidad y selectividad necesarias, así como la rápida transitoriedad de los NPS en el mercado. Además, se exploran también los beneficios e inconvenientes de los diferentes modos de adquisición y exploración de los datos para mejorar la capacidad de identificación con especial énfasis en IMS y las herramientas de predicción previamente presentadas en esta tesis.

Contents

List of acronyms

1,25-D3	1α,25-dihydroxyvitamin D3
2C-E	2,5-dimethoxy-4-ethylphenethylamine
3,4-DiMeO-α-PVP	3,4-dimethoxy-α-pyrrolidinopentiophenone
4-C-α-PPP	4-chloro-α-pyrrolydinopropiophenone
4-FMC	4-fluoromethcathinone
4-MEC	4-methylethcathinone
4-MePPP	4-methyl-α-pyrrolydinopropiophenone
5-IT	5-(2-aminopropyl)indole
ACN	Acetonitrile
AhR	Arylhydrocarbon receptor
AIF	All-Ion fragmentation
AMT	α-methyltryptamine
ANN	Artificial Neural Networks
APCI	Atmospheric pressure chemical ionization
AR	Androgen receptor
AR-	Antagonistic androgen receptor
AR+	Agonistic androgen receptor
bbCID	Broadband collision induced dissociation
BE	Benzoylecgonine
BEQ	Bioanalytical equivalent concentration
bk-DMBDP	Dimethylpentylone
bk-MDDMA	3,4-methylenedioxy-N,N-dimethylcathinone
BS	Procedural blank sample
CCS	Collision cross section
CEC	Chemical of emerging concern
CERAPP	Collaborative estrogen receptor activity prediction project
CHI	Chromatographic hydrophobicity index
CID	Collision induced dissociation
DDA	Data-dependent acquisition mode
DEHP-*d*$_3$	Di(ethylhexyl)phthalate-*d*$_3$
DHT	Dihydrotestosterone
DI	Direct injection
DIA	Data-independent acquisition mode
DT	Drift time

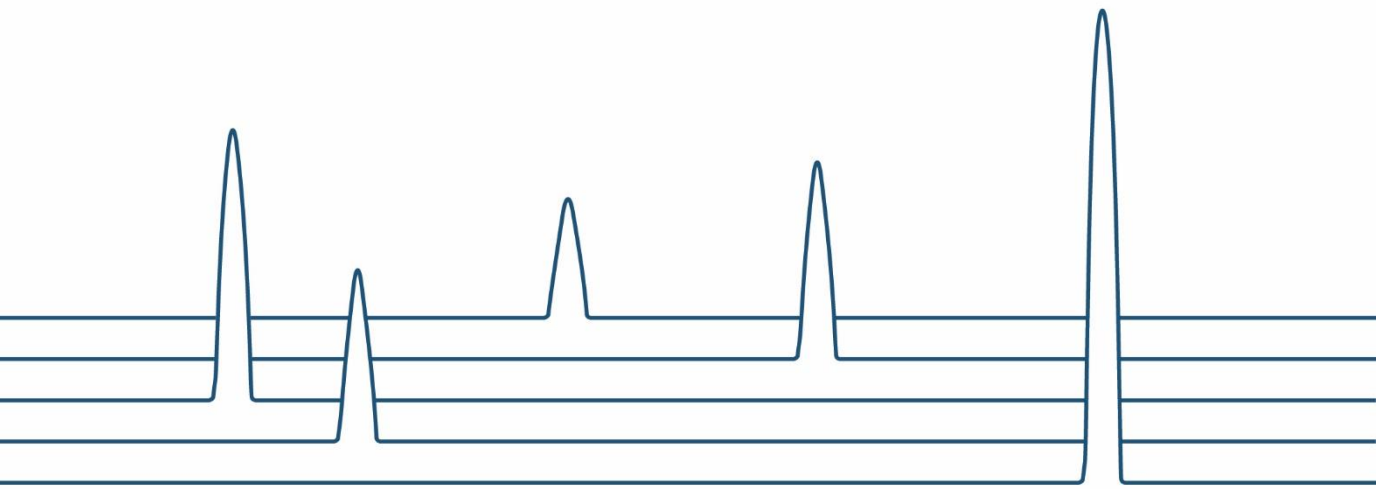
DTIMS	Drift tube ion mobility separation
E2	17β-estradiol
EBT	Effect-based trigger value
EC	effect concentration (*e.g.* EC_{50}: sample REF value to produce 50% of the maximum effect produced by positive control)
EDA	Effect-directed analysis
EF	Enantiomeric enrichment fraction
EFLEA	*N*-hydroxy-*N*-methyl-3,4-ethylenedioxyamphetamine
EMCDDA	European monitoring centre for drug and drug addiction
ER	Estrogen receptor
ER-	Antagonistic estrogen receptor
ER+	Agonistic estrogen receptor
ESI	Electrospray ionization source
EWS	Early warning system
EWW	Effluent wastewater
FTIR	Fourier transform infrared spectrometer
FWHM	Full width at half maximum
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
HCOOH	Formic acid
HDMS^E	High definition MS^E
HE	High energy function
HepG2	Human hepatocellular carcinoma
HILIC	Hydrophilic Interaction Liquid Chromatography
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
ID	Illicit drug
ILD	Instrumental limit of detection
ILIS	Isotopically labelled internal standards
ILRS	Isotopically labelled reference standards
IMS	Ion mobility separation
IMS-HRMS	Ion mobility separation coupled to high resolution mass spectrometry
IP	Identification point
IT	Ion trap mass analyzer

IWW	Influent wastewater
K	Ion's mobility
LC	Liquid chromatography
LC-HRMS	Liquid chromatography coupled to high resolution mass spectrometry
LC-IMS-QTOF MS	Liquid Chromatography coupled to ion mobility separation and hybrid quadrupole-time of flight mass spectrometry
LE	Low energy function
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LRMS	Low resolution mass spectrometry
LW	Lake water
m/z	Mass-to-charge ratio
MARS	Multiple adaptive regression splines
MCX	Mixed-mode cationic exchange
MDHOET	3,4-methylenedioxy-*N*-hydroxyethylamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MDPV	Methylenedioxypyrovalerone
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^E	MS-to-the-E. Data-independent acquisition mode for Waters QTOF MS instruments
NH_4Ac	Ammonium acetate
NMR	Nuclear magnetic resonance
NPLC	Normal phase liquid chromatography
NPS	New psychoactive substance
Nrf2	Nuclear factor erythroid 2-related factor 2 (oxidative stress response)
NTS	Non-target screening
OECD	Organization for economic cooperation and development
OHF	Hydroxyflutamide
OMP	Organic micropollutant
PCP	Personal care product
PET	Polyethylene terephthalate

PFASs	Perfluoroalkyl substances
PMMA	ρ-methoxymethamphetamine
PU	Pooled urine
Q	Quadrupole mass analyzer
QqQ	Triple quadrupole mass analyzer
QSRR	Quantitative structure-retention relationship
QTOF	Hybrid quadrupole time-of-flight mass analyzer
R^2	Correlation coefficient
R	Mass spectrometric resolution
Ral	Raloxifene
REF	Relative enrichment factor
RP	Reverse phase
RPLC	Reverse phase liquid chromatography
RS	Reference standards
RSD	Relative standard deviation
RT	Retention time
RTi	Retention time interpolation scale
RTI	Retention time indexing system
RTi-N	Retention time Interpolation scale in negative mode
RTi-P	Retention time interpolation scale in positive mode
S/N	Signal-to-noise ratio
SCORE	Sewage analysis CORe group Europe
SFC	Supercritical fluid chromatography
SI	Supporting information
SLE	Solid-liquid extraction
SMILES	Canonical simplified molecular line entry system strings
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring mode
SS	Suspect screening
SST	System suitability test
SVM	Support vector machines
SVR	Supported vector regression
SW	Surface water
SWATH	Sequential window acquisition of all theoretical fragment ion spectra mode

tBHQ	tert-butylhydroquinone
TCDD	2,3,7,8-tetrachlorodibenzodioxin
THC-COOH-*d*$_3$	11-Nor-9-carboxi-delta-9-tetrahidrocannabinol-*d*$_3$
TIMS	Trapped ion mobility separation
TOF	Time-of-flight mass analyzer
TP	Transformation product
TS	Target screening
TWIMS	Travelling wave ion mobility separation
UHPLC	Ultra-high performance liquid chromatography
UHPLC-IMS-MS	Ultra-high performance liquid chromatography coupled to ion mobility separation and mass spectrometry
UHPLC-MS	Ultra-high performance liquid chromatography coupled to mass spectrometry
UHPLC-MS/MS	Ultra-high performance liquid chromatography coupled to tandem mass spectrometry
UNODC	United nations office on drugs and crime
VDR	Vitamin D receptor
VDR-	Antagonistic vitamin D receptor
VDR+	Agonistic vitamin D receptor
VHC	Vehicle control
WBE	Wastewater based epidemiology
WHO	World health organization
WW	Wastewater
WWTP	Wastewater treatment plant
XIC	Extracted ion chromatogram
α-PVP	α-pyrrolidinopentiophenone
µLC	Micro liquid chromatography
µLC-MS/MS	Micro liquid chromatography coupled to tandem mass spectrometry

OBJECTIVES



Objectives

The **main objective** of this thesis was to investigate the analytical capabilities of state-of-the-art liquid chromatography coupled to ion mobility separation (IMS) and mass spectrometry for the analysis of organic micropollutants (OMP) in the aquatic environment as well as the refinement of current target, suspect and non-target screening strategies. For this purpose, different instrumental configurations have been used based on tandem mass spectrometry, using triple quadrupole (QqQ) and quadrupole-time of flight (QTOF) instruments as well as travelling wave IMS coupled to QTOF MS.

A **3-step research strategy** was followed: *(a)* identification of the analytical problem which needs to be solved or the research gap which needs to be further explored in order to provide more comprehensive data, *(b)* development of strategies, tools and/or methodologies able to address the identified problem in an accurate and resolutive way, and *(c)* application of the developed tools in real samples.

Following this strategy, several **specific objectives** were set up to achieve the general goal of this thesis:

i. Evaluation of the utility of collision cross section values from IMS for the target screening of OMPs in complex matrices. Development and application of an identification criteria for small molecules by means of ultra-high performance liquid chromatography coupled to ion mobility separation and high resolution mass spectrometry (UHPLC-IMS-HRMS).

ii. Investigation on the impact of including IMS data in HRMS-based target and suspect screening workflows. Investigation of its impact on spectral quality and false positive detections as well as evaluation of the robustness of CCS measurements.

iii. Development of a retention time interpolation strategy for liquid chromatography to compensate for analyte retention time shifting due to matrix interferences in target and suspect screening approaches.

iv. Exploration of new statistical tools to develop and validate *in silico* prediction models for retention time and/or CCS for compounds whose

reference standard is not available at the time of analysis. Application in suspect screening strategies of small molecules in complex matrices.

v. Evaluation of a strategy based on effect-directed analysis (EDA) for a comprehensive water quality assessment. Integration of chemical and biological analyses in terms of observed toxicities and identified OMPs.

vi. Identification of the main hurdles associated with the analysis of a particularly challenging group of chemicals (new psychoactive substances, NPS) in influent wastewater and pooled urine.

vii. Development, validation and application of a targeted methodology for the quantitative determination of selected NPS in influent wastewater by means of solid-phase extraction and UHPLC coupled to tandem MS (triple quadrupole).

viii. Application of the previously generated knowledge to highlight the main perspectives and strategies to tackle the monitoring of NPS through wastewater and pooled urine analysis.

Objetivos

El **principal objetivo** de esta tesis fue el estudio de las capacidades analíticas de instrumentación de última generación para cromatografía líquida acoplada a separación por movilidad iónica (IMS) y espectrometría de masas para el análisis de contaminantes orgánicos (OMP) en el medio acuático, así como el perfeccionamiento de las actuales estrategias de cribado dirigido, cribado de sospechosos y cribado no dirigido. Con este fin, se han utilizado diferentes configuraciones instrumentales basadas en espectrometría de masas en tándem, triple cuadrupolo (QqQ) y cuadrupolo-tiempo de vuelo (QTOF) así como IMS de onda viajera acoplada a QTOF MS.

Se ha seguido una **estrategia de investigación** de 3 pasos: (*a*) identificación del problema analítico que necesita ser resuelto o el nicho de investigación que necesita ser explorado con mayor profundidad para proveer de datos completos, (*b*) desarrollo de estrategias, herramientas y/o metodologías para tratar el problema identificado de forma precisa y resolutiva, y (*c*) aplicación de la herramienta desarrollada en muestras reales.

Siguiendo esta estrategia, se establecieron varios **objetivos específicos** que permitieran llegar al objetivo principal de la tesis:

i. Evaluación de la utilidad de los valores de sección transversal de colisión (CCS) de IMS para el cribado dirigido de OMPs en muestras complejas. Desarrollo y aplicación de un criterio de identificación para moléculas pequeñas mediante el uso de cromatografía líquida de ultra alta resolución acoplada a separación por movilidad iónica y espectrometría de masas de alta resolución (UHPLC-IMS-HRMS).

ii. Investigación sobre el impacto de incluir datos de IMS en cribados dirigidos y no dirigidos con HRMS. Investigación del impacto en la calidad espectral y el número de detecciones de falsos positivos, así como evaluación de la robustez en la medida de valores de CCS.

iii. Desarrollo de una estrategia de interpolación de tiempo de retención para cromatografía líquida con el objetivo de compensar las derivas en

el tiempo de retención de los analitos cuando estos se ven afectados por interferentes presentes en la matriz de la muestra.

iv. Exploración de nuevas herramientas estadísticas para desarrollar y validar modelos de predicción computacionales de tiempo de retención y/o CCS para aquellos compuestos cuyo patrón de referencia no está disponible en el momento del análisis. Aplicación en estrategias de cribado de sospechosos para moléculas pequeñas en matrices complejas.

v. Evaluación de una estrategia basada en análisis dirigido a efectos (EDA) para un control exhaustivo de la calidad del agua medioambiental. Integración de análisis químico y biológico en términos de toxicidad observada y OMPs identificados.

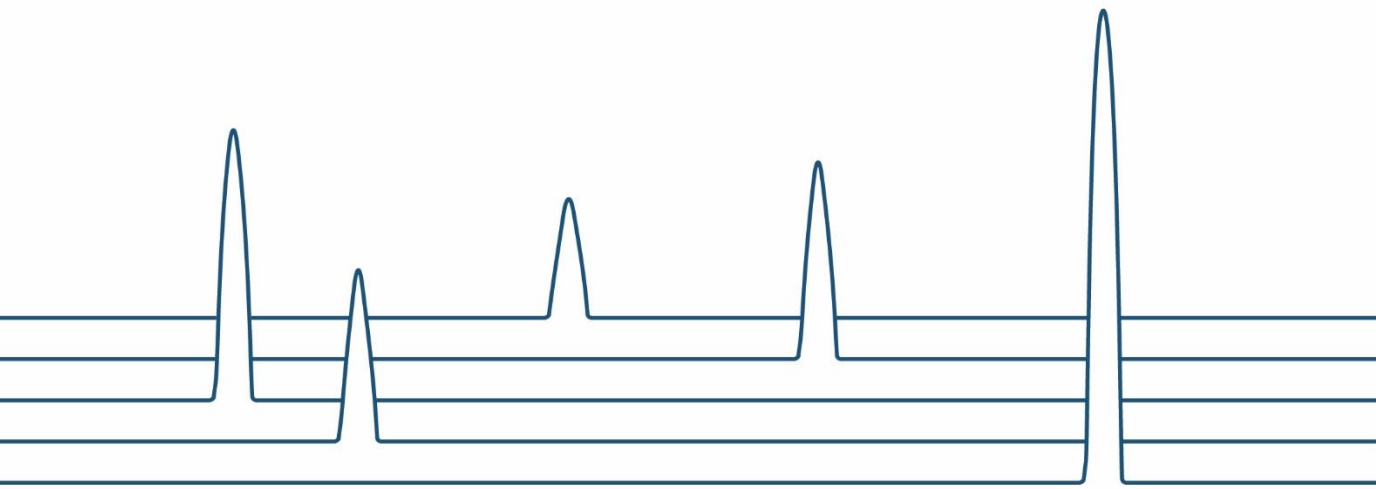
vi. Identificación de los principales retos asociados con el análisis de un grupo de sustancias particularmente complicado (nuevas sustancias psicoactivas, NPS) en muestras de agua residual influente y orina.

vii. Desarrollo, validación y aplicación de una metodología dirigida para la determinación cuantitativa de NPS seleccionados en agua residual influente mediante extracción en fase sólida (SPE) y UHPLC acoplada a espectrometría de masas en tándem (triple cuadrupolo).

viii. Aplicación del conocimiento previamente generado para destacar las principales estrategias y perspectivas para enfrentar el control del consumo de NPS mediante el análisis de aguas residuales y orina.

CHAPTER 1.

GENERAL INTRODUCTION



Chapter 1. General Introduction

1.1. Impact of human activity on the aquatic environment

1.2. Analytical techniques for monitoring of aquatic environment

1.2.1. Chromatographic separation

1.2.1.1. Liquid chromatography

1.2.2. Ionization sources

1.2.3. Mass spectrometry

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1.3. Screening strategies for the monitoring of the aquatic environment

1.3.1. Target, Suspect and Non-Target screening strategies

1.3.2. Online and home-made databases

1.3.3. *In-silico* prediction tools

1.3.4. Reporting confidence in OMP and CEC identification

1.1. Impact of human activity on the aquatic environment

Environmental pollution is not a new phenomenon. In reality, it has been accompanying humankind ever since the first gatherings of humans into stable settlements for a prolonged period of time (Markham, 1994). **Environmental pollution** can be understood as the release of any substance or any form of energy to the environment at a rate faster than it can be dispersed, diluted, decomposed, recycled, or safely stored (Nathanson, 2021). Therefore, pollution did not become an environmental problem until permanent human settlements burgeoned into large populations. Ever since, the release of either physical or chemical farming, manufacturing, industry and population waste products to the environment has been threatening the surrounding ecosystems and, as a consequence, compromising the environmental sustainability (Markham, 1994).

Although pollution can also be originated from natural events such as forest fires or active volcanoes, the main sources of polluting agents are of anthropogenic origin. And, therefore, environmental pollution has evolved in parallel to the evolution of humankind from the unintentional self-poisoning with heavy metals in the Paleolithic to the uncontrolled disposal of wastewater in medieval towns and the widespread utilization of pesticides in modern agriculture.

However, it was not before the 19th century when humanity started to raise concerns about environmental pollution and understood the prominent role of unhealthy living conditions and water contamination for the spread of diseases. From this knowledge, big metropolises such as Chicago took measures to control wastewater discharges and built the first large sewage system connected to a wastewater treatment plant of the United States in mid-1850s (**Figure 1.1**) (National Ocean Service (US), 2021). After that, other big cities followed their example and develop wastewater treatment facilities. However, awareness of a crystal clear relation between environmental pollution and health took more time to be gained. Severe and acute episodes such as the Great Smog of London in 1952 (**Figure 1.1**) extraordinarily helped to move towards a deeper understanding of the effects of environmental pollution in public health. In this particular incident, the combination of a dense fog with low temperatures and elevated concentrations of NO_2 and SO_2 due to the coal combustion for energy and heat production resulted in the generation of deathly high concentrations of sulfate aerosols in the air of the city of London. This is estimated to have been responsible of killing approximately 12,000 people (Martínez, 2021; Wang et al., 2016). In our current days, environmental factors are also silently responsible of a massive number of deaths. The World Health Organization (WHO) estimates that 24% of the global deaths occurred in 2016 can be somehow related to modifiable environmental factors and around 22% of global diseases were triggered by environmental pollution (Prüss-Üstün et al., 2016).

The presence of organic micropollutants of anthropogenic origin in the aquatic environment is ubiquitous and can also be related to a high percentage of the pollution-linked deaths. **Organic micropollutants** (OMPs) can be defined as chemicals of anthropogenic origin that occur in the aquatic environment above a natural basal level because of human activities but with concentrations still remaining at trace levels (Stamm et al., 2016). The definition of OMPs encompasses many different chemical classes based on their application: pesticides, pharmaceuticals, personal care products (PCPs), illicit drugs (ID) and new psychoactive substances (NPS), detergents, hormones, industrial chemicals, mycotoxins, flame retardants, perfluoroalkyl substances (PFASs), etc.

Figure 1.1. *(a)* Layout of sewer system planned to be built in Chicago at the end of 1857 (extracted from National Ocean Service (US), 2021); *(b)* Trafalgar Square in London on December 5th of 1952 during the Great Smog of London (extracted from Stevens, 2012).

The total amount of OMPs yearly released to the environment is incommensurable and, therefore, several unexpected acute episodes of toxicity due to the presence of OMP have been reported in the literature (Prüss-Üstün et al., 2016). These events are often prompted by non-appropriate water sanitation measures where population is exposed to drinking water polluted with unknown levels of OMPs. For example, over 30,000 pesticide intoxications were observed in Jiangsu Province (China) along the period 2007-2016 with more than 1,700 fatalities due to contaminated water originated from agricultural activities (Wang et al., 2019). However, environmental pollution does not only affect human beings but also other organisms. Recently, Tian *et al.* identified a so far unknown OMP responsible for an enhanced mortality of Coho salmon in urban creeks in northwest US affected by roadway runoffs and stormwater. Therein, a transformation product of an ubiquitous tire rubber antioxidant was found to be at toxic levels and, therefore, affecting the survival of salmon individuals (Tian et al., 2021). The different idiosyncrasies of these examples demonstrate the fact that aquatic environmental pollution and it consequences for the surrounding ecosystems evolved in parallel to the evolution of humankind and new contaminants are constantly emerging.

In this sense, although there is no yet agreed definition, **chemicals of emerging concern** (CECs) can be described as newly identified chemicals whose widespread use or omnipresent fate in the aquatic environment in the past few decades has raised concern, either because analytical limitations have hitherto hindered their finding or due to their still unknown potential impact in the fauna, flora and human welfare of the surrounding environment (Nilsen et al., 2019; Sauvé and Desrosiers, 2014). Overall, OMPs and CECs are widespread in the environment and their effects over different organisms of the surrounding ecosystems is often unknown. In this regard, different legislations to regulate the utilization of certain chemicals, to ban the application of particular pesticides and to monitor the fate of OMPs and CECs in the environment have been promulgated by authorities, *e.g.* the 'Watch List' for chemicals in environmental water samples (European Commission, 2020; Joint Research Centre of European Commission et al., 2015). From this perspective, analytical chemistry is pivotal

to monitor the occurrence of OMPs and CECs in the environment and provide data to decision makers over chemicals identified in the environment so they can make accurate and science based decisions.

1.2. Analytical techniques for monitoring of aquatic environment

Over the recent decades, many efforts have been made to provide reliable data on the fate of OMPs in the aquatic environment. With the outstanding improvement in both analytical techniques and strategies, different classes of OMPs have arisen to be monitored (Hernández et al., 2019; Richardson and Kimura, 2016). Currently, there are many different chemical classes that can be encompassed under the definition of OMP as abovementioned. Even that, OMP was traditionally limited to a reduced number of groups of chemicals such as pharmaceuticals, pesticides or personal care products. However, other chemical groups were eventually incorporated and, currently, the list of chemical groups within the umbrella of OMP is massive (Hernández et al., 2019; Richardson and Kimura, 2016; Wilkinson et al., 2017). In this thesis, research has been focused on pharmaceuticals and metabolites, pesticides and transformation products (TPs), hormones, mycotoxins, PCPs, PFASs, and special attention has been devoted to lifestyle biomarkers such as ID and NPS.

Different strategies have been proposed in the literature to monitor OMPs in aquatic environmental samples (Brack et al., 2016; Brunner et al., 2020; Vergeynst et al., 2015). However, the mostly implemented analytical approach for the identification, monitoring and evaluation of OMPs in environmental samples is the combined utilization of chromatographic separation and mass spectrometry (MS) (Hernández et al., 2019; Hollender et al., 2017; Krauss et al., 2010; Menger et al., 2020; Schymanski et al., 2015). More recently, different studies have been published incorporating ion mobility separation (IMS) and, therefore, expanding the possibilities of the analytical strategies (Bijlsma et al., 2019; Hinnenkamp et al., 2019; Regueiro et al., 2016). The conjunction of these techniques permits to gather sensitivity, selectivity and identification power to confirm compound identity in complex matrices.

Before going into more details about the analytical techniques, special attention should be drawn to the **sample preparation** strategies. Provided that the nature of screening strategies is to monitor the presence of a wide range of OMPs in environmental samples, sample preparation should be kept to a minimum to avoid any potential analyte losses during this step. Accordingly, different strategies, *e.g.* direct injection (DI), have been tested for the screening of OMPs in environmental samples (Boix et al., 2015; Fonseca et al., 2020; Vergeynst et al., 2014). These strategies have been proven of use for certain groups of chemicals with concentrations high enough to be detected with current instrumentation. However, to monitor low concentrations of OMPs in complex samples, pre-concentration approaches are often preferred (Menger et al., 2020). Liquid-liquid extraction (LLE), solid-liquid extraction (SLE), solid-phase extraction (SPE), passive sampling and solid-phase microextraction (SPME) are examples of different sample treatment techniques. In parallel to the fact that analytes of interest are being concentrated, these techniques also permit the removal of matrix-endogenous compounds that might complicate the posterior sample analysis. Therefore, their application is especially beneficial when dealing with difficult matrices such as surface water or influent wastewater (Menger et al., 2020; Pourchet et al., 2020). In this thesis, SPE has been the technique of choice for the analysis of different OMPs in environmental samples, due to the extraordinary potential for pre-concentration of analytes as well as clean up technique to remove matrix endogenous interferents. In brief, SPE is able to retain target compounds by their interaction with mobile and stationary phases. Depending on the physicochemical properties of target analytes, different stationary phases can be selected such as weak or strong anion exchange, weak or strong cation exchange, and hybrid hydrophilic-lipophilic balance (HLB). The latter is able to retain compounds of interest from different polarities and, therefore, cover a wider range of compounds of different chemical classes. For this reason, it is often the preferred sorbent for SPE sample treatments in screening strategies of environmental samples.

Despite sample treatment is often kept at its minimum for screening analyses, it is often required for an appropriate performance of the overall screening

strategy. Therefore, it should also be carefully thought-out during the study design. Frequently, environmental water samples are analyzed by means of combining SPE with chromatography coupled to mass spectrometry. In the following sections, an in-depth description of the analytical techniques used in this thesis is explained.

1.2.1. Chromatographic separation

Chromatography embraces a group of different techniques that enable the separation, identification and determination of components of complex matrices (Skoog et al., 1998). To this purpose, a sample is transported by a mobile phase through an immiscible stationary phase where a repeated mass transfer process involving adsorption-desorption occurs. Then, different types of chromatography can be defined depending on the nature of the mobile phase, which can be a gas (gas chromatography, GC), a liquid (liquid chromatography, LC) or a supercritical fluid (supercritical fluid chromatography, SFC) (Skoog et al., 1998). By selecting appropriate mobile and stationary phases, analytes of interest that are present in the sample distribute between phases at different degrees. As a result, compounds strongly retained in the stationary phase will elute from the chromatography later than compounds poorly retained and, thus, with higher affinity for the mobile phase (Skoog et al., 1998).

An appropriate distribution of compounds between stationary and mobile phases in GC is accomplished for thermostable and volatile compounds in the range of low to non-polarity. Contrarily, LC can cover a wider range of polarities including compounds in the range of low to high polarity and non-volatile solvents (such as water) can be used.

In the particular context of monitoring OMPs in water samples, the vast majority of compounds of interest are of low to high polarity and, therefore, LC-amenable. Additionally, the nature of the samples strongly facilitates their analysis by LC. Overall, LC is the technique of choice for the analysis of OMPs in the aquatic

environment, although there are some studies using GC to monitor particular groups of contaminants.

LC has been applied across the different studies presented in this thesis to refine screening strategies for a comprehensive monitoring of the aquatic environment.

1.2.1.1. Liquid chromatography

Liquid chromatography utilizes the distinctive affinity of the components of the sample mixture, contained and transported by means of a liquid mobile phase, with the sorbent particles of the stationary phase (Skoog et al., 1998). Depending on the distinct polarities of the compounds of interest, different types of LC separation techniques can be employed:

i. **Normal phase LC (NPLC).** In NPLC, polarity of the stationary phase is higher than that of the mobile phase and, therefore, polar compounds are more retained than non-polar compounds (Skoog et al., 1998). The utilization of non-polar organic solvents such as hexane makes NPLC non-compatible with mass spectrometric systems. Consequently, its application for environmental monitoring is negligible.

ii. **Reverse phase LC (RPLC).** With non-polar stationary phases, RPLC is by far the most used LC technique due to the possibility of using aqueous mobile phases mixed with organic solvents to obtain moderate-high polar mobile phases. Polar analytes are less retained in the stationary phase due to a higher affinity for the mobile phase and, as a consequence, RPLC enables the analysis of highly polar to low-polar chemicals (Skoog et al., 1998). Due to the increased range of compound polarities that can separated by means of RPLC as well as the huge variety of stationary phase chemistries commercially available, it is often the technique of choice for the analysis of OMPs in environmental samples. Moreover, mobile phase buffers can be used to improve chromatographic performance. However, when coupled to a

mass spectrometer, special attention should be paid to avoid the utilization of non-volatile additives that could precipitate in the ionization source resulting in decreased signal intensities.

iii. **Hydrophilic interaction LC (HILIC).** High polar stationary phases are used for HILIC with aqueous mobile phase combined with polar organic solvents. Therefore, polar compounds are more retained in the stationary phase, permitting the early elution of non-polar compounds (Nguyen and Schug, 2008). It is, therefore, ideal for the analysis of high polar analytes when coupled to mass spectrometric systems due to the possibility of using volatile organic solvents. Due to its relative novelty, HILIC has still been scarcely applied for the monitoring of OMP in environmental samples (Nguyen and Schug, 2008).

In this thesis, RPLC has been the separation technique evaluated.

According to the Van Deemter equation, other chromatographic parameters such as stationary phase particle size and mobile phase flow rate mainly affect the performance of the chromatographic separation (Skoog et al., 1998). **High performance LC** (HPLC) and **ultra-high performance LC** (UHPLC) have revolutionized the LC technique with enhanced separation by reducing the particle size from traditional LC columns (~10 μm) (Skoog et al., 1998). HPLC columns are packed with particles with diameter between 3.5 – 5.0 μm. However, the range of flow rates for an optimal separation is limited. Columns with even smaller particle size are used in UHPLC separations. With particles <2.0 μm in diameter, faster separations are permitted without compromising the separation power of the technique due to wider range of optimal flow rates. With such small particles, very narrow chromatographic peaks can be obtained and, as a consequence, chromatographic resolution and sensitivity are substantially increased (López-Ruiz et al., 2019). However, LC pumps able to stand the high back-pressures resulting from the UHPLC columns are required as well as fast scanning MS instruments able to comprehensively detected narrow chromatographic peaks.

In the particular field of environmental aquatic samples, the main interest is to have well resolved and high intense chromatographic peaks that enable an appropriate identification by means of MS instruments. Therefore, UHPLC is the main LC separation technique nowadays applied.

From a different perspective, but also pursuing to improve chromatographic performance, **micro fluidic chromatographic techniques** have been developed. Commonly, UHPLC uses flow rates in the sub-mL min^{-1} range; nevertheless, flow rates in the range of few µL min^{-1} are used in micro-liquid chromatography (µLC) or nL min^{-1} in nano-flow chromatography. The drastic reduction in the flow rate used in µLC in comparison to UHPLC together with the associated and required reduction in the internal diameter of chromatographic column are responsible for an enhanced sensitivity (Fanali et al., 2013; King et al., 2018). **Figure 1.2** depicts the sensitivity improvement factor observed for a series of small organic molecules when using different types of micro fluidic chromatography in comparison to conventional UHPLC analysis. With column internal diameter of µLC around 150 µm and flow rates in the range of 1 – 5 µL min^{-1}, the improvement factor observed for µLC is in the range x4 – x30. The main benefit of µLC is accomplished when coupled to MS instruments, due to an enhanced ionization efficiency in the ionization source (Murphy et al., 2014).

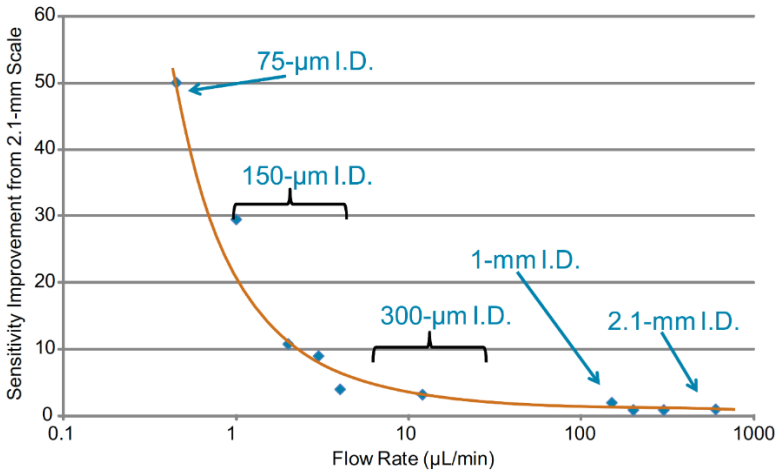


Figure 1.2. Average sensitivity improvement with reducing column diameter and flow rates in comparison to conventional UHPLC columns for a series of small molecules. (Extracted from Murphy et al., 2014)

Therefore, it allows to reach lower limits of detection and limits of quantification in comparison to conventional UHPLC coupled to MS (Kleinnijenhuis et al., 2016). However, chromatographic run time is often larger than in conventional UHPLC and, because of the smaller dimensions, sample composition may compromise the analyte separation in μLC systems (Fanali et al., 2013; Kleinnijenhuis et al., 2016).

The abovementioned advantages of μLC remark microfluidic chromatographic separation as a potential improvement for the analysis of especially low concentrated species in complex matrices. However, scarce applications are reported in the literature due to its novelty. In this thesis, μLC coupled to MS was evaluated for its potential application to monitor NPS consumption through wastewater analysis (**scientific article 9** of **chapter 5**).

1.2.2. Ionization sources

One of the biggest challenges when coupling an LC system to a mass spectrometer has been the proper generation of ionized species from the compounds eluting from chromatography. Over the years, different interfaces have been developed but the approaches that are mostly used are atmospheric pressure chemical ionization (APCI) and, **electrospray ionization** (ESI). By far, ESI is the ionization source with a wider level of implementation in research laboratories for environmental applications. It enables the analysis of thermolabile compounds of a wide range of molecular weights and it is considered the most suitable ionization source for polar or even ionic compounds. Accordingly, it is also the ionization source used in the different studies presented in this thesis.

In the ESI interface (**Figure 1.3**), the flow of mobile phase containing the analytes of interest moves through a capillary set at a certain voltage (generally 1 – 3 kV). At the exit of the capillary, the elevated voltage together with the action of a nebulizer gas disperses the liquid flow into a fine aerosol of small charged droplets (nebulization). These droplets, which are under atmospheric pressure

conditions, are heated up by a desolvation gas flow at elevated temperature. Thus, solvent is evaporated from a charged droplet until it becomes unstable upon reaching the Rayleigh limit. In this moment, droplets explode into smaller charged droplets due to the enhanced electrostatic repulsion of charges following a process known as the Coulomb explosion. Those small droplets undergo successive processes of solvent evaporation and Coulomb explosion until gas-phase ions are created by leaving the analytes with the charges (Dass, 2007; Hiraoka, 2013). Then, ionized species are attracted to the off-axis entrance of the MS system by a charged cone walled by a gas in counter-flow. Such orthogonal distribution and the counter flow gas help to minimize the introduction of non-charged species into the vacuumed system. Afterwards, a series of transfer lens in step-alike disposition (StepWave transfer system in Waters Corporation instruments) extract ionized species from the gas flow and introduce them in the MS analyzer. In this way, ion beam is again refined from non-ionized species or remaining mobile phase solvent. Therefore, chemical noise can be reduced and, thus, signal to noise ratio (S/N) increased.

ESI interface can work either in positive ionization mode where a molecule receives a proton to form $[M+H]^+$ (mostly basic molecules), or in negative ionization mode where a molecule releases a proton and forms $[M-H]^-$ (mostly acidic molecules). However, other species such as sodium ($[M+Na]^+$), potassium ($[M+K]^+$) or ammonium ($[M+NH_4]^+$) adducts in positive ionization mode and

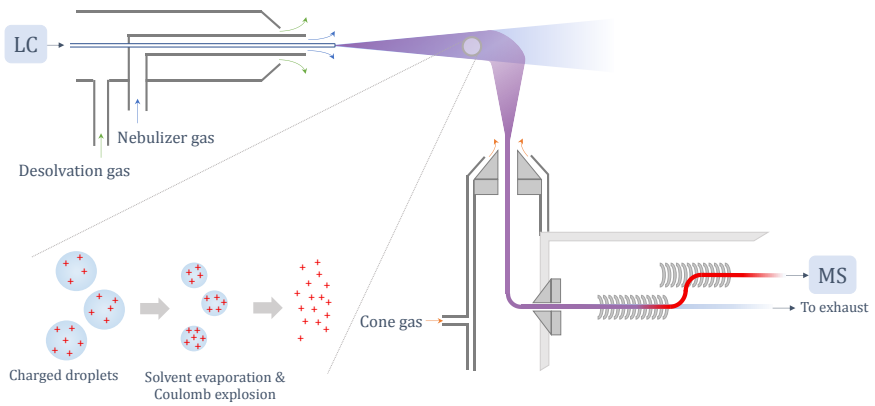


Figure 1.3. Schematic view of Z-orthogonal ESI ionization source as implemented in Water Corporation instruments.

chloride ($[M+Cl]^-$) or formate ($[M+HCOO]^-$) adducts in negative ionization mode can also occur.

ESI is the most used ionization source for LC coupled to MS instruments. However, the combination of μLC with ESI is challenging because of dead volumes playing an important role at the micro-scale. Recently, **integrated μLC-ESI chip MS interfaces** have been developed and greatly reduced the problems related to dead volumes in laboratory-assembled μLC equipment (Aggarwal et al., 2015; Fanali et al., 2013; King et al., 2018). Such integrated interfaces join into the same device the μLC column and ESI (**Figure 1.4**) and, therefore, eliminates the possibility of misassembling instrumental parts. In these devices, such as the *iKey* for ionKey/MS systems from Waters Corporation, the ionization efficiency is higher. The electrospray plume reduces in size because of the reduced mobile phase flow and, therefore, sampling efficiency in the electrospray source is greatly improved (Murphy et al., 2014).

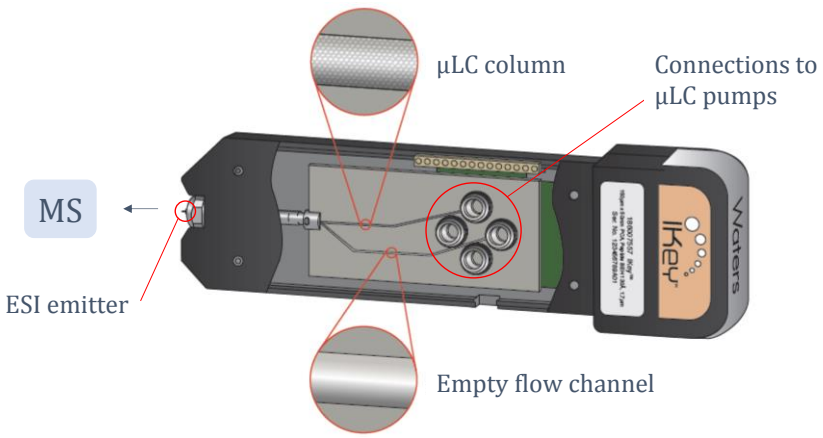


Figure 1.4. Schematic view of an *iKey* device from Waters Corporation (extracted and modified from Doneanu and Donegan, 2016).

It is, however, noteworthy that co-eluting compounds may alter the electrospray ionization process and lead to the suppression or (less frequently) enhancement of the analyte signal. This phenomenon is known as **matrix effect**. While there is no accepted hypothesis to explain ion enhancement, ion suppression results from the competition of co-eluting species in the formation of charged droplets.

The occurrence and intensity of matrix effect is highly variable with many influencing factors such as physicochemical properties of the analyte, matrix composition, sample treatment and chromatography. Therefore, several strategies have been proven useful to correct for matrix effect such as improvement of sample treatment, sample dilution or, more preferably, the utilization of isotope labelled internal standards (ILIS) as surrogates. In this thesis, the latter has been used in the quantitative determination of NPS in WW samples (**scientific article 9** of **chapter 5**).

1.2.3. Mass Spectrometry

Since its first development more than 100 years ago, MS has become one of the most powerful techniques for the analysis and identification of organic compounds in complex matrices. Mass spectrometry is an analytical technique able to measure the molecular mass of individual gas-phase ions by separating them on the basis of their mass-to-charge (*m/z*) ratios, either in time or space. (Dass, 2007; Hiraoka, 2013). Different types of mass spectrometers arise based on the different mass analyzers that can be used, such as quadrupole (Q), ion trap (IT), time-of-flight (TOF) and Orbitrap.

The different performance of the mass analyzers can be characterized by several parameters: resolution, mass accuracy, efficiency, mass range, liner dynamic range, speed and sensitivity. Yet, resolution (R) is maybe the most differential one. In MS, R is defined as the ability to distinguish two peaks of neighboring *m/z* values. This allows MS instruments to be divided into two different categories: **low resolution MS** (LRMS) and **high resolution MS** (HRMS). The choice of which is the most appropriate MS category as well as mass analyzer to be used mainly depends on the purpose of the study.

The combination of more than one mass analyzer into a single instrument has contributed to improve sensitivity and selectivity as well as to provide an enormous amount of information for identification purposes. These tandem MS (MS/MS) instruments are by far the most widely applied and are a powerful

technique for the qualitative and quantitative analysis of OMPs in complex matrices. Among the large set of instrumental configurations, the studies presented in this thesis have been carried out by means of MS/MS instruments of both LRMS (triple quadrupole, QqQ) and HRMS (hybrid quadrupole time-of-flight, QTOF).

1.2.3.1. Triple Quadrupole mass analyzer

Triple quadrupole (QqQ) is a tandem LRMS instrument that consists of two quadrupole mass analyzers (Q) in series with a quadrupole in between (q) acting as a collision cell. The collision cell is usually filled with an inert gas (usually Ar) to force the collision between the gas molecules and an ion previously selected in the first quadrupole (Q_1). Those collisions provoke that precursor ions fragment into product ions. This process is known as collision-induced dissociation (CID). Then, product ions traverse the second quadrupole (Q_2) before reaching the detector (Dass, 2007). Currently available QqQ instrumentation does not always consist of a quadrupole as collision cell. Different instrumental improvements have been conducted in the collision cell towards the reduction of the time necessary to generate and release product ions (*i.e.* reducing dwell time). In the case of the triple quadrupole instrument used in this thesis, it features a *ScanWave* collision cell consisting of a series of lens where waves of energy can be applied. Therein, ions are fragmented, accumulated and sequentially released at high speed.

Both quadrupoles can work either in Full Scan mode or Selected Ion Monitoring (SIM) mode. By combining these two working modes between both Q_1 and Q_2, four different analysis modes depicted in **Figure 1.5** arise:

i. **Product Ion Scan.** The Q_1 isolates an ion with an established *m/z* value (precursor ion) that is afterwards fragmented in the collision cell (**Figure 1.5a**). Then, Q_2 is set up to scan all ions within a range to monitor product ions generated from the precursor selected. This

working mode provides information of whole fragmentation spectra for a single compound which is valuable for elucidation purposes.

ii. **Selected Reaction Monitoring (SRM).** In this mode, Q_1 selects an ion based on the *m/z* value which is then fragmented in the collision cell (**Figure 1.5b**). After that, Q_2 permits only to a particular product ion to traverse and reach the detector. In this way, only certain product ion from a particular precursor is detected.

iii. **Precursor Ion Scan.** The Q_2 selects only a product ion that can reach the detector while Q1 is working under full scan mode (**Figure 1.5c**). This set up is particularly useful for the selective identification of closely related classes of chemicals in mixtures.

iv. **Neutral Loss Scan.** Both quadrupoles (Q_1 and Q_2) are scanned at the same time, but with a constant mass offset (**Figure 1.5d**). This allows to recognize ions which suffer from a loss of a neutral molecule in the

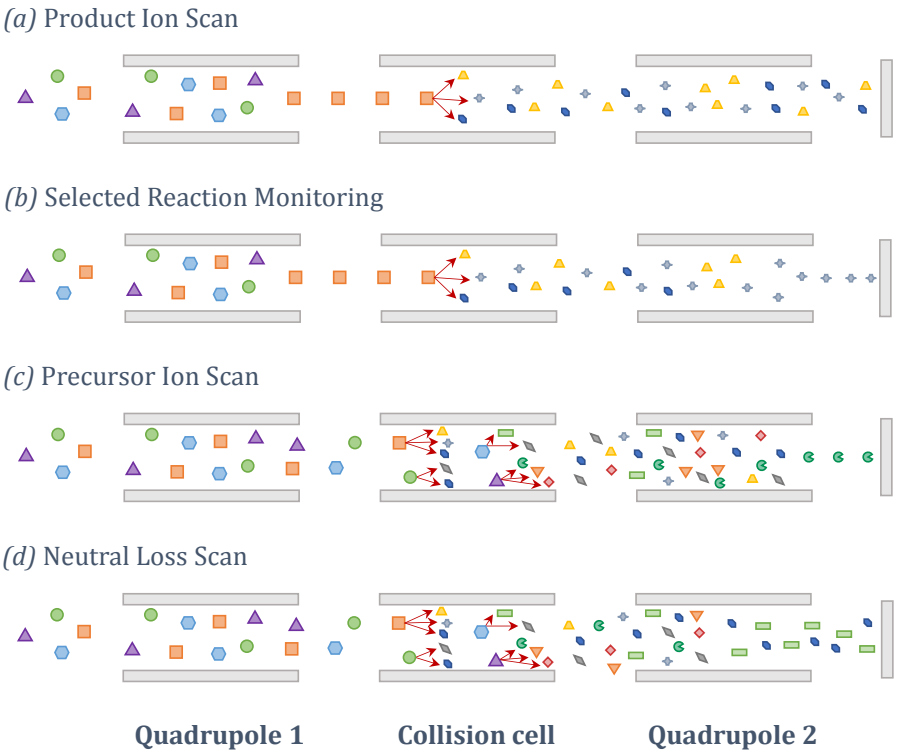


Figure 1.5. Scheme of QqQ working modes: (a) product ion scan, (b) selected reaction monitoring, (c) precursor ion scan and (d) neutral loss scan.

collision cell (*e.g.* H_2O, NH_3, CO). This configuration enables particular functional groups released in the CID to be monitored giving also information about the *m/z* value of precursor ions with those functional groups.

Among all the possibilities offered by QqQ, SRM is by far the most widely applied working mode. It offers high sensitivity and selectivity due to the removal of background noise and, as a consequence, enhanced S/N ratio (Dass, 2007). In addition, powerful instruments capable of very short dwell times (1-5 ms) can monitor hundreds of SRM transitions per second. Therefore, SRM can be used for the determination and quantification of numerous compounds in a single analysis which substantially reduces analyses time. However, their utilization for qualitative determination is limited by the relatively low resolution (approximately 1 Da) and low sensitivity in scan mode. In this thesis, QqQ has been explored for the determination of low concentrations of NPS in wastewater.

1.2.3.2. Hybrid quadrupole time-of-flight mass analyzer

High resolution mass spectrometers are by far the most versatile and powerful MS instruments currently available. The high mass resolving power and mass accuracy as well as the sensitive full spectrum acquisition poses HRMS instruments as one of the most used instrument in research laboratories to monitor OMPs and identity new CECs in environmental samples. In this thesis, hybrid QTOF has been applied.

Hybrid QTOF is based on the combination of a quadrupole and a TOF mass analyzers (**Figure 1.6**). Briefly, TOF mass analyzers consist on a flight tube where ions are separated based on their different velocities. To their purpose, ions are accelerated by an energy pulse in the pusher. Such energy is transformed in kinetic energy, making the ions fly through the flight tube at different velocities depending on their *m/z* value. Therefore, the time that it takes for an ion to reach the detector at a known distance is unique since heavier ions reach lower speed than lighter ones. The flight tube has a 'V' shape because

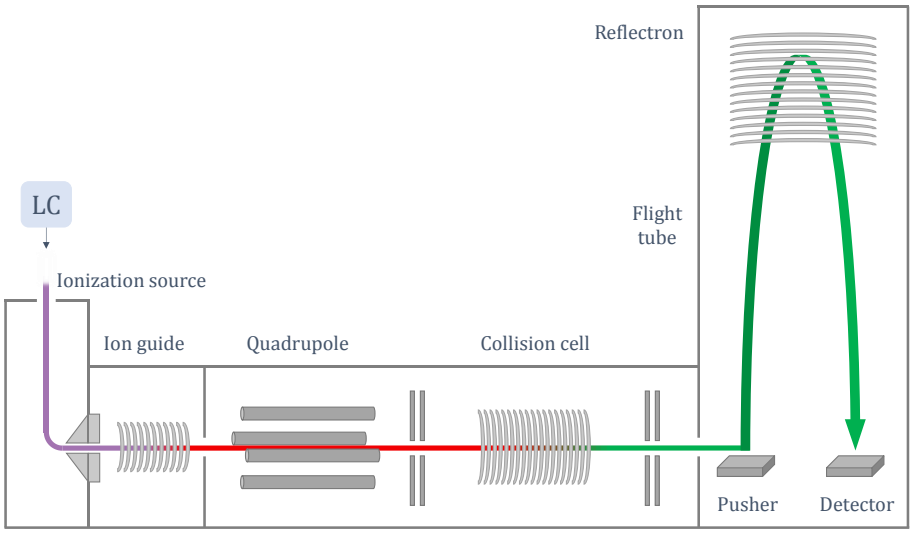


Figure 1.6. Hybrid Q-TOF scheme based on a XEVO G2 QTOF from Waters Corporation.

the reflectron is used to focus ions with the same *m/z* value and, as a consequence, they arrive simultaneously at the detector (Dass, 2007; Hiraoka, 2013). This provides an improvement in mass resolution and permits to measure ions at accurate mass. The TOF instrument used in this thesis shows a mass resolving power higher than 40,000 at full width at half maximum (FWHM) at *m/z* 556.

Hybrid QTOF MS features a single quadrupole, a collision cell and TOF mass analyzer (as depicted in **Figure 1.6**) and can provide even more possibilities for screening and identification. They permit the acquisition of accurate mass full-scan spectra of (de)protonated molecules (with the quadrupole acting as an ion guide with no ion resolution) as well as obtaining relevant structural information of product ions at accurate mass. The latter is enabled by performing MS/MS analyses and selecting the precursor ion in the first quadrupole (Dass, 2007). However, pre-selection of ions in MS/MS experiments may limit the potential of QTOF instruments since other contaminants not previously selected can be ignored (even if they are present at high concentrations) and, therefore, incur a false negative. Thus, combining MS and MS/MS acquisitions in a single injection is of special interest to take full advantage of hybrid QTOF MS. In this sense,

depending on the working mode of the first quadrupole, two different acquisition modes arise:

i. **Data dependent acquisition (DDA).** In DDA, both full scan spectra and MS/MS data are sequentially acquired. In the first, quadrupole works under non-resolving conditions so that TOF can analyze all ions. Next, the quadrupole is set up as a mass-resolving quadrupole, where a precursor ion is selected to be afterwards fragmented at the collision cell (operating as described for QqQ mass analyzer). Then, product ions resulting from the fragmentation are analyzed by means of TOF providing mass accurate full-spectrum of product ions. Precursor ions can be previously selected (inclusion list) or automatically selected by the instrument if an ion occurs at an intensity higher than a pre-established value or other previously established criteria in the prior full scan acquisition.

ii. **Data independent acquisition (DIA).** In Waters instruments, as used in this thesis, DIA mode is branded as MS^E acquisition mode. In MS^E, the quadrupole is operated as an ion guide with no pre-selection of precursor ions. MS^E methods are based on the sequential acquisition of full-scan spectra at different collision energies. At first, a full-scan spectrum is acquired for all ions eluting from chromatography at a **low collision energy** (LE). Almost simultaneously, energy in collision cell ramps to a high energy value so molecules are fragmented into product ions and a full-scan spectrum at **high collision energy** is acquired (HE). Therefore, sequential information of intact ionized molecules and their fragments is gathered in a single injection. DIA permits processing after acquisition as well as reprocessing with a different strategy. Thus, it enables the retrospective analysis of samples for previously not considered or unknown OMPs.

In this thesis, QTOF instrument has been mainly operated under MS^E conditions in order to permit the application of different analytical strategies explained in following sections.

1.2.4. Ion Mobility Separation

Ion mobility separation studies the ion characteristics such as shape and size by observing their response upon an acceleration in a buffer gas by means of an electric field (Donald and Prell, 2019). A wide variety of instrumental configurations for IMS analysis exist; however, the most interesting ones for this thesis are those hyphenated to an MS instrument, which open a window of new possibilities (Gabelica and Marklund, 2018; Lee, 2017).

Having an extra physical separation orthogonal to chromatography and MS provides relevant and meaningful information for the identification of small molecules. Although LC and MS themselves provide information about RT and accurate mass for precursor and product ions, IMS incorporates to the equation the value of the shape and size of the ions monitored.

In brief, IMS separates ions based on their mobility (K) which is defined in conventional IMS instruments as the ratio of its average velocity in the direction of the applied electric field to the amplitude of the field (Donald and Prell, 2019; Lee, 2017). When operating under low electric field amplitude and high buffer gas pressure, the **collision cross section** (CCS) of a molecule, *i.e.* the relationship between an ion's mobility and its size, can be described by means of the Mason-Schamp equation (Donald and Prell, 2019; Mason and Schamp, 1958). According to Mason and Schamp, CCS is a function of the mass of the ion and its charge, temperature and K. However, operating IMS under Mason-Schamp-like conditions is challenging when coupled to LC systems because of the large analysis time required to derive an ion's mobility (Gabelica et al., 2019). Certainly, different developments have pushed forward the hyphenation of LC-HRMS instruments with IMS.

Enhanced mobility separation technologies with analysis time in the millisecond scale make modern IMS-HRMS instruments compatible with chromatographic separations. By the application of functionalized electric fields in the mobility cell, faster separations are accomplished in a smaller space. Therefore, different instrumental configurations appeared such as the drift tube IMS (DTIMS, from Agilent), trapped IMS (TIMS, from Bruker) or the travelling wave IMS (TWIMS,

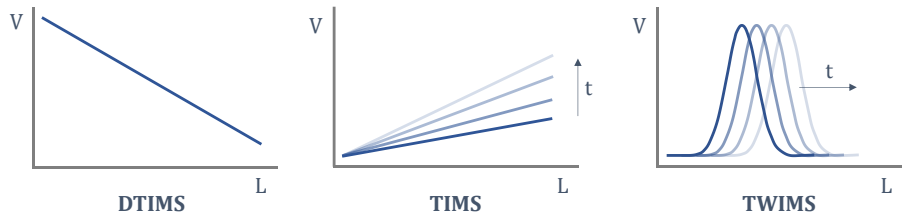


Figure 1.7. Different functions of electric field *vs.* length of mobility device in commercially available IMS-MS instruments. Fade in color indicates movement of the curve in time.

from Waters) (**Figure 1.7**). They all differ in the functionalization of the electric field along the mobility cell length (Gabelica et al., 2019; Gabelica and Marklund, 2018; Lee, 2017). While DTIMS applies a constant function over time for the electric field, TIMS and TWIMS apply sequential distribution of electric potential as a way to push ions through the mobility device. In this thesis, the instrumental configuration used corresponds to a Vion QTOF from Waters Corporation. This instrument works under TWIMS technology.

In **TWIMS instruments**, ions are separated by means of consecutive electric field waves moving in the direction of the electric field (Shvartsburg and Smith, 2008). As depicted in **Figure 1.8**, ionized molecules from the ionization source arrive at the mobility cell, where they are trapped in small groups. Those lots are then pushed by the electric field through a mobility gas, usually N_2 (although He is also popular for other instrumental configurations). The electric pulses in TWIMS work similar to sea waves where a single one wave suffice to bring back a small and light beach ball to the sand, whereas a bigger and heavier ball will require several sea waves to traverse the same distance. That is the principle applied for TWIMS, where small and light ions travel faster through the mobility device than big and heavy ones which require several travelling electric field waves (Shvartsburg and Smith, 2008). After being separated by IMS, ions encounter the same instrumental distribution and configuration abovementioned for a QTOF.

The separation provided by IMS can be measured by means of computing the time that it takes for an ion to transit the mobility device (*i.e.* drift time, DT). Thus,

after appropriate instrument calibration, DT can be translated into CCS values (Shvartsburg and Smith, 2008). CCS (measured in $Å^2$) represents the cross-section of the sphere created by the ion when moving in the mobility device and, therefore, it is a unique value for each molecule that can be afterwards used as an additional identification parameter alongside RT and MS data.

Since TWIMS instruments are based in QTOF mass analyzers, similar acquisition modes to those previously explained are available. Both DDA and DIA can be applied and incorporate a new dimension to the data set generated (4-D: RT, CCS, MS and intensity). In the same way as conventional QTOF, for TWIMS-QTOF instruments the most popular acquisition mode is DIA, called **High Definition MS^E** (HDMS^E). In this acquisition mode, LE and HE functions are acquired sequentially as well as DT is measured for all ions. With the aid of sophisticated peak deconvolution tools, LE and HE data can be componentized into features with information of RT, CCS for (de)protonated molecule, in-source fragments and adducts, as well as HE fragments.

Several benefits arise from the utilization of UHPLC-IMS-HRMS instruments such as low background noise, additional identification parameter accomplished by CCS calculation, and mass spectral cleaning due to interferences removal. It is

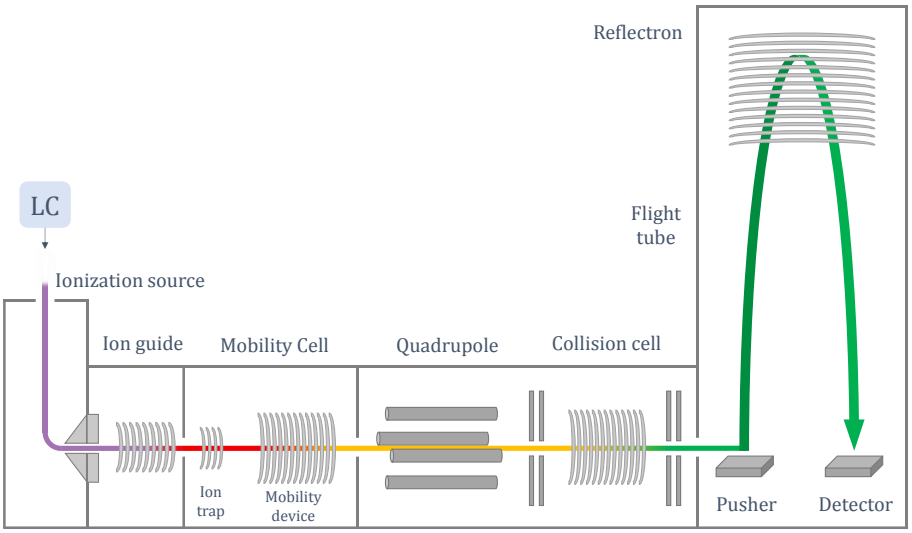


Figure 1.8. Schematic view of a VION QTOF from Waters Corporation as used in this thesis.

therefore the most promising technique for the implementation in the screening of OMPs in complex matrices. Several research articles in this thesis have been carried out under these premises.

1.3. Screening strategies for the monitoring of the aquatic environment

The wide range of possibilities offered by hyphenated UHPLC-MS and UHPLC-IMS-MS have fostered the development of comprehensive approaches for monitoring the occurrence of OMPs and CECs in the aquatic environment (Hernández et al., 2019; Hollender et al., 2017; Krauss et al., 2010; Menger et al., 2020; Schymanski et al., 2015). The distinct instrumental configurations available as well as their variety of working modes occasioned the rise of numerous approaches, each of them with their own goals, advantages and drawbacks. The instrument to be applied as well as the working mode for its mass analyzers should be, therefore, decided based on the aim of the study.

As abovementioned, LRMS can provide an elevated sensitivity and selectivity which can be of utility for the screening of low concentrations of compounds in complex matrices. However, the optimal performance of LRMS is achieved when working under SRM mode and, therefore, preselecting the compounds of interest. Thus, there is no possibility to expand the scope of the study. Contrarily, if the study aims to routinely monitor an established list of contaminants, the application of LRMS is the most suitable approach in terms of sensitivity, selectivity, dynamic range and costs. Hence, LRMS instruments are generally used for the quantification of targeted OMP, although targeted screening strategies with qualitative purposes can also be implemented.

From a different perspective, the utilization of hybrid HRMS instruments is more versatile and offers new possibilities for the screening of organic compounds. The acquisition of full-spectrum data gives highly valuable information, which allows retrospective analysis, the discovery of unreported CECs or wide-scope screening analyses of large lists of compounds.

1.3.1. Target, Suspect and Non-Target screening strategies for High Resolution Mass Spectrometry

Irrespective of whether hybrid HRMS instruments are operated under DDA or DIA acquisition modes, distinct data-processing approaches can be employed depending on the purpose of the study:

i. **Target screening (TS)**. TS is performed with information about RT, CCS as well as MS data on (de)protonated molecule and fragment ions (Schymanski et al., 2014a) gathered during previous analysis of the compounds of interest. It is, therefore, necessary to have reference standards. As a consequence, TS is often limited by the commercial availability of compounds as well as their associated cost. This type of analysis is directed towards the analysis of a specific list of substances previously selected. Therefore, the solely goal of the analysis is to monitor these targeted compounds with no attention for other substances that might be present in the sample (Krauss et al., 2010; Menger et al., 2020). TS can be performed by means of both DDA and DIA acquisition modes. For DDA, an inclusion list of the target compounds needs to be set up based on the previously acquired data (*i.e. m/z* for (de)protonated molecule). Whereas, in the case of DIA, data can be processed after acquisition for the list of the target compounds. In both cases, prior knowledge of RT and fragmentation extraordinarily facilitates the identification process.

ii. **Suspect screening (SS).** Suspect analysis is also directed towards a list of compounds but, in this case, no reference standard is available and, as a consequence, there is no previously acquired empirical data for them. Similarly to TS, both DDA (by means of an inclusion list) or DIA acquisition modes can be implemented for SS strategies. For this strategy, *m/z* values of chemicals suspected to be present in the sample (suspects) are compared to measured *m/*z observed in the sample. Therefore, for SS only a list of substances including chemical formula (and so, *m/z* value) is necessary (Krauss et al., 2010; Menger et al., 2020). However, since no previous information on RT and empirical

fragmentation is available, the inclusion of third-party data such as reported fragment ions can be of great utility for data processing. SS is by far the most often applied screening strategy due to its versatility and the possibility to search for large lists of chemicals in a single injection making use of sophisticated software and processing algorithms.

iii. **Non-target screening (NTS).** Non-target screening is definitely the most complex and time-consuming strategy. With full spectrum acquisition and no preselection of compounds enabled by both DDA and DIA, NTS aims to find unknown or previously unreported compounds (Krauss et al., 2010; Menger et al., 2020). Thus, the complexity of environmental samples makes NTS somehow a never-ending story due to the elevated number of substances potentially presented in the sample. As a consequence, feature prioritization strategies based on frequency of detection, associated toxicity, etc. can be implemented to facilitate the analysis. Additionally, analysts with a profound knowledge on compound identification, organic chemistry and mass spectrometry is pivotal for NTS strategies.

As abovementioned, both DDA and DIA can be implemented for TS, SS and NTS. Yet, each of them have their own pros and cons. In the case of DDA, high quality MS/MS data is obtained for selected ions (either based on an inclusion list or when an intensity threshold is exalted) and, therefore, data interpretation is facilitated. However, the number of substances that can be analyzed by DDA in a single injection is limited by *(i)* the speed at which full scan data is inspected by the intelligent software algorithm searching for ions matching the inclusion criteria, and *(ii)* the time necessary for ions to be isolated, fragmented and analyzed. Nonetheless, DIA analyses can monitor an unlimited number of substances in a single injection and, thus, enable TS and/or SS analyses of thousands of compounds without the necessity of reinjecting the sample. Yet, fragmentation spectra provided by DIA is of less quality because of coeluting interferents being also fragmented. While this has been a limitation for DIA, the hyphenation of IMS to hybrid HRMS instruments has extraordinarily improved

the quality of DIA fragmentation spectra due to the enabled DT alignment. In this thesis, DIA by means of IMS-HRMS has been mainly used.

The most powerful and comprehensive approach is to combine TS, SS and NTS into a single analysis. In this sense, known OMPs with previously acquired data, OMPs for which no reference standards are available as well as their TPs and metabolites, and still unknown CECs can be screened in a single injection.

In order to assure a good performance of any of the strategies followed, there are some tools to be considered such as the availability of online and home-made databases, and the application of prediction models that can support tentative identifications. The following sections of this introduction will give some insight on the usefulness of these tools for TS, SS and/or NTS. Finally, the criteria to report the confidence on the identification of compounds will also be discussed.

1.3.2. Online and home-made databases

The availability of curated and comprehensive databases of OMPs is becoming feasible, and also pivotal, with the increased capabilities of modern instruments and processing software to analyze thousands of chemicals in a single injection.

In the ideal case, large databases with information of target compounds of interest should be developed. Those databases should encompass information on RT, (de)protonated *m/z* value and MS fragmentation for the instrumental configuration to be used in the analysis. Also, CCS could be incorporated if IMS is used. Yet, developing in-house target databases is time-consuming and expensive; and, as a consequence, having large and curated ones is non-affordable for many research groups. However, it is essential for an appropriate performance of TS of OMPs and CECs (Hernández et al., 2019; Krauss et al., 2010) and, therefore, many open access initiatives by the scientific community are flourishing.

Nevertheless, there are many chemicals of interest for which reference standards could not be available at the laboratory and, therefore, third-party data can also be of interest. In this sense, in-house suspect databases can be

developed by incorporating information previously reported by other researchers in scientific literature. Thus, information on expected *m/z* and fragments can be compiled and used for the finding of organic contaminants by means of SS (Hernández et al., 2019; Krauss et al., 2010; Menger et al., 2020). This type of database is especially relevant for the monitoring of TPs and/or metabolites due to the often lack of commercially available reference standards. Therefore, only information on the *m/z* value needs to be incorporated. Additionally, in-house SS databases can be populated with information found in mass spectral online databases.

In order to facilitate the implementation of SS approaches as well as to favor the access to third-party data, different online databases have been developed over the last years. Under these premises, the NORMAN network established in 2015 the NORMAN Suspect List Exchange platform (https://www.norman.network.com/nds/SLE/), where network participants can upload their in-house target or suspect databases (NORMAN network, 2021). Therefore, it is a collection of lists of compounds that strongly facilitates data exchange and use information. Also, online spectral databases have been built. The latter, with information on expected *m/z* and fragmentation, serve as a repository of data where anyone interested can check the empirical data gathered by colleagues for the suspected compound under study to increase the confidence on their identification or to discard the candidate. The most popular online spectral database might be MassBank, whose European brand is constructed and populated also under the framework of the NORMAN network (https://massbank.eu/MassBank/).

Also noteworthy is the case of HighRes NPS, a collaborative online database where researchers can report newly detected NPS along with their empirical data gathered from both reference standards or seized substances (Mardal et al., 2019) in exchange to access other researchers' data. It is, therefore, a combination of both compound and spectral database since it contains information on newly detected NPS and, if available, empirical data. HighRes NPS contains information of more than 2,000 psychoactive substances (HighRes NPS Community, 2021).

In the particular case of CCS data, the relative novelty of the hyphenation of IMS to HRMS makes the availability of empirical CCS data in online databases still scarce. Nonetheless, there are some tools recently developed that are devoted to share empirical CCS data. As an example, AllCCS by Zhou *et al.* (http://allccs.zhulab.cn) contains information on more than 3,500 empirical CCS values (Zhou et al., 2020). Another approach is presented by Ross *et al.* in CCSbase (https://ccabase.net) where they collect data published by other researchers into a CCS data hub (Ross et al., 2020). Although these are examples of online CCS databases, the availability of IMS data for small molecules and particularly for OMPs is still limited.

With the trend to move towards an open access science with equality of opportunities to use scientific data generated, the development, improvement and utilization of online databases is pivotal.

1.3.3. *In-silico* prediction tools

As shown, one of the biggest limitation to the improvement of SS performance is often the lack of a reference standard and/or the high economical cost to build target databases. If it is commercially available, the problem might be solved by firstly checking online databases as well as a posterior purchase for confirmation. However, there are cases, such as metabolites, TPs or newly reported CECs, for which previously reported empirical data might be limited or even unattainable. Therefore, predictive algorithms can be very helpful.

There are relevant topics for environmental analysis by LC-MS or LC-IMS-MS that can be anticipated. Firstly, ***in-silico* fragmentation predictors** can give assistance in order to explain the fragmentation observed for a putative identification and, as a consequence, make a decision on the identification. In this sense, MetFrag is a well-known tool for environmental analytical chemists that aids in the comparison of predicted mass spectral data with empirical one by means of specialized software (Ruttkies et al., 2016; Wolf et al., 2010). However,

there are other *in-silico* fragmentation tools that can be of great help in SS such as MassFragment (Yu et al., 2008), MS-Finder (Tsugawa et al., 2016), etc.

Other approaches have aimed to predict **transformation products or metabolites** by predicting which reactions might occur depending on the chemical structure such as BioTransformer (Djoumbou-Feunang et al., 2019), University of Minnesota – Pathway prediction system (Ellis et al., 2008), or Meteor Nexus (Judson et al., 2015). These tools are very important for the study of by-products of OMPs in the environment which can pose an even higher hazard than the original compound. By predicting potential TPs or metabolites, SS lists can be built and used to search for their presence in the aquatic system.

From a different perspective, **RT and CCS predictive tools** have also been developed. The instrument-dependent nature of RT have resulted in an elevated number of predictive models by different mathematical algorithms (Aalizadeh et al., 2019; Bade et al., 2015b, 2015a; Barron and McEneff, 2016; Mollerup et al., 2018; Stanstrup et al., 2015; Yang et al., 2020; Yeung et al., 2020). However, their utility relies on the same principle. By comparing empirical and predicted RT for a suspect candidate, the analyst can have a rough idea of the RT window where the structure under study should be eluting. For CCS, despite the still scarce availability of online databases, several CCS predictive models have been proliferating (Bijlsma et al., 2019; Colby et al., 2019; Gonzales et al., 2016; Mollerup et al., 2018; Plante et al., 2019; Ross et al., 2020; Zhou et al., 2017, 2016). Recent studies over the reproducibility of CCS measurement over different instruments highlighted that CCS predictive models can be easily incorporated into other laboratories provided that certain conditions are fulfilled such as IMS technique and/or CCS calibrants used (France et al., 2020; Hinnenkamp et al., 2018; Righetti et al., 2020; Stow et al., 2017).

The main objective of predictive tools is to gather more information and, consequently, confidence on the identification of chemicals of interest. Thus, the combined utilization of different predictive models is very recommended (Bijlsma et al., 2019). However, in order to achieve the highest level of confidence into the potential identification of an OMP, reference standards need to be

purchased and analyzed under the same conditions. Nonetheless, predictive tools can be of help to prioritize and reduce costs of buying different reference standards for the same finding.

1.3.4. Reporting confidence in OMP identification

Due to the different possibilities offered by state-of-the-art HRMS and IMS-HRMS instruments to identify organic compounds in environmental samples, there is a need to have a common protocol to report data. Also, having well-defined and widely accepted criteria by the scientific community is essential to harmonize dissemination of results so that different studies can be compared.

Several studies have proposed different identification confidence levels for HRMS studies (Blaženović et al., 2018; Herrera-Lopez et al., 2014; Nácher-Mestre et al., 2016; Rivier, 2003; Rochat, 2017; Schymanski et al., 2014a; Sumner et al., 2007). However, the most widely accepted classification was proposed by Schymanski *et al.* with a 5-level criteria for the identification of small molecules using chromatographic separation coupled to HRMS (**Figure 1.9**) (Schymanski et al., 2014a). In this classification, mass spectrometric features can be classified from level 5 – exact mass of interest (lowest confidence) to level 1 – confirmed structure (highest confidence). For the latter, reference standard is required to match the empirical data gathered and represents the ideal scenario. Besides, different levels are established for tentative identifications with no reference standard (levels 2 and 3). In those levels, putative identifications are based on the gathering of information from other sources such as previously reported data or online databases as well as the existence of unequivocal diagnostic fragments. Depending on whether there is only a single structure likely to match the empirical data (level 2) or more than one structure that can fit (level 3), levels for tentative identifications are differentiated. Additionally, level 4 and 5 highlight the importance of marking recurrent findings over different samples even if only a molecular formula or exact mass can be collected. But, no mention to IMS data is done in such classification.

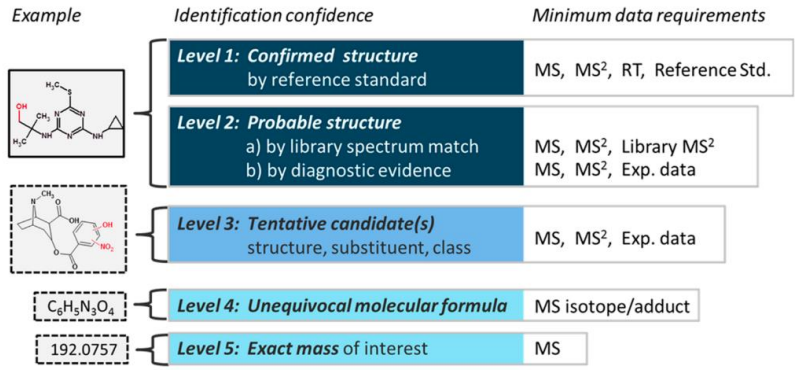


Figure 1.9. Identification confidence levels by Schymanski et el. (2014). (Extracted from Schymanski et al., 2014a)

Different studies have also been devoted to incorporate CCS as an additional identification parameter into the identification criteria (Bauer et al., 2018; Goscinny et al., 2019, 2015; Lian et al., 2018; Monge et al., 2019; Nuñez et al., 2019; Regueiro et al., 2016; Tejada-Casado et al., 2018). So far, the most extensive approach to propose IMS inclusion into identification criteria was made by Monge *et al.* where they incorporated IMS into previously reported classification by Sumner *et al.* (Monge et al., 2019; Sumner et al., 2007). However, no discussion on the minimum accuracy requirements was made and no instructions on how to apply the criteria were reported. Thus, such classification has not been widely accepted by scientific community. Consequently, curated and carefully thought out levels were still to be proposed for the inclusion of IMS.

The way in which identification confidence is reported in environmental screening of OMPs is pivotal for a reliable assessment of pollution. Therefore, widely accepted criteria should be pursued so that the whole scientific community can better understand the actual situation of pollution of aquatic environment.

1.4. Scientific articles included in this thesis

The core of this doctoral thesis consists of 10 scientific articles published (or submitted) in peer-reviewed journals within the first quartile of the ISI Web of

Knowledge. It is significant to emphasize that several articles herein included have been completed in close collaboration with recognized researchers who pertain to well-known and prestigious international research institutes.

In **Chapter II**, the hyphenation of IMS to LC-HRMS as an additional separative dimension is discussed. Such hyphenation arises several benefits for the improvement of TS, SS and NTS strategies that are depicted and explained in **scientific article 1** and **2**. Additionally, the inclusion of IMS into widely accepted identification criteria for small molecules is proposed in **scientific article 1**, where for the first time a thorough discussion on how to apply the criteria is reported. Additionally, a target database for 556 OMPs is presented for LC-IMS-HRMS instruments.

Chapter III is devoted to the development of refinement tools for SS and NTS approaches. In the search for a robust and reliable measure of retention behavior of organic molecules, **scientific article 3** shows the development of a RT interpolation system aiming to correct for RT shifting in complex matrices. Furthermore, *in-silico* predictive tools for the calculation of CCS (**scientific article 4**) and CCS and RT (**scientific article 5**) are described. As discussed in these articles, their utilization in SS analyses is essential.

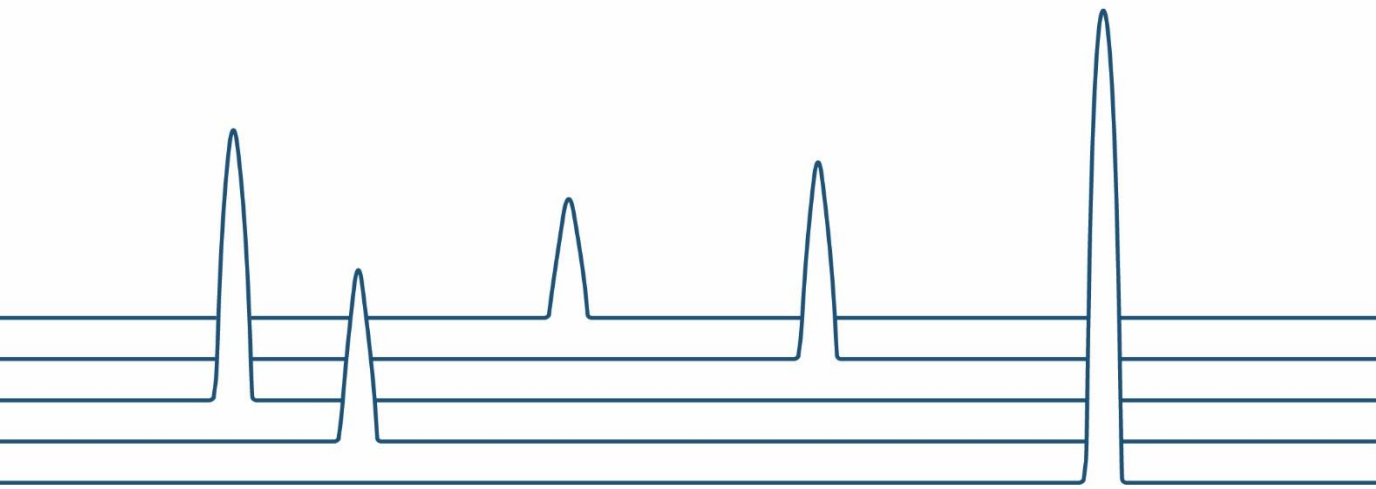
Then, **Chapter IV** explores a more comprehensive strategy to monitor environmental pollution by the combination of chemical and biological analyses. In **scientific article 6**, a bioanalytical study of the toxicity fingerprint of different surface water samples from Spanish protected wetlands is done. Afterwards, chemical analysis was performed in the same sample set to search for potential linkage between toxicity and the presence of OMPs. The results of the latter are presented in **scientific article 7**. It is important to remark that these two articles resulted from a pre-doctoral research stay at the Swedish University for Agricultural Sciences (SLU) in Uppsala (Sweden) under the supervision of Dr. Johan Lundqvist, Dr. Lutz Ahrens and Prof. Karin Wiberg.

Finally, **chapter 5** is devoted to a special group of organic molecules of interest. In this chapter, the monitoring of NPS consumption through wastewater analysis is investigated. In **scientific article 8**, an in-depth review of the studies and

methodologies published over the previous years is presented. Next, a targeted methodology for the monitoring of a set of different NPS in wastewater by means of LRMS is presented in **scientific article 9.** Also, the potential of μLC-MS/MS for the improvement of sensitivity capabilities of targeted methodology is evaluated. Finally, **scientific article 10** summarizes the principal perspectives and challenges of NPS monitoring in wastewater samples. This article makes a review of the different methodologies to be used and also serves as a good overview of the different approaches and strategies applied throughout this thesis for the monitoring of small organic molecules in complex matrices.

CHAPTER 2.

THE HYPHENATION OF ION MOBILITY SEPARATION TO LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY AS AN ADDITIONAL SEPARATION DIMENSION



Chapter 2. The hyphenation of Ion Mobility Separation to Liquid chromatography-Mass Spectrometry as an additional separative dimension

2.1. Introduction

2.2. Scientific Articles

2.2.1. Scientific article 1.

"Improving target and suspect screening high resolution mass spectrometry workflows in environmental analysis by ion mobility separation"

Environmental Science & Technology 54 (2020) 15120-15131

2.2.2. Scientific article 2.

"Ion mobility separation in LC-HRMS opens new screening strategies for chemicals of emerging concern in the aquatic environment"

Chemosphere 280 (2021) 130799

2.3. Discussion of Results

2.1. Introduction

Over the last decade, HRMS instruments paved the way for outstanding developments in the field of target, suspect and non-target screening of organic micropollutants (OMP) in environmental analyses (Hernández et al., 2019; Hollender et al., 2017; Schymanski et al., 2015). By offering a powerful alternative to former targeted screening methodologies by means of LRMS, HRMS have become the technique of choice for a wide range of studies (Bletsou et al., 2015; Ibáñez et al., 2014; Knolhoff and Croley, 2016; Leendert et al., 2015; López et al., 2016). In addition to the high resolution and mass accuracy, the large variety of acquisition modes and parameter settings available (DDA, DIA, windowed-DIA, …), have fostered the implementation of HRMS-based screening workflows (Alygizakis et al., 2018; Brack et al., 2019; Schymanski et al., 2014b). Generally, HRMS instruments are operated under accurate-mass full-spectrum acquisition modes that enable to screen for an almost unlimited number of substances (Hernández et al., 2019; Schymanski et al., 2015). However, the data generated (comprising information of RT, *m/z* and peak intensities) under these acquisition modes, especially in DIA analyses, is complex and large. Therefore, its evaluation can be challenging and time-consuming. Moreover, environmental matrices are typically of high complexity with OMPs being found at low

concentrations. Therefore, their analysis often translates into challenging compound identifications because of potential chromatographic RT deviations and mass spectral interferences resulting in false positive/negative detections (Alygizakis et al., 2018; Hernández et al., 2019; Menger et al., 2020; Pourchet et al., 2020).

In this sense, hyphenation of IMS with LC-HRMS instruments represents an innovative tool for target and untargeted screening approaches that might help to overcome some of the abovementioned issues associated with environmental screening by means of conventional LC-HRMS (D'Atri et al., 2018; Dodds and Baker, 2019; Eldrid and Thalassinos, 2020; Morrison and Clowers, 2019). The addition of drift time (DT) as an extra dimension to the obtained chromatographic RT and accurate mass results in an increased selectivity as well as an improved identification, especially for DIA modes (Bijlsma et al., 2019; Regueiro et al., 2016). After chromatographic separation and ionization, coeluting ions are separated in the mobility device, which enables (de)protonated molecules and their fragment ions of interest with the same DT to be aligned. Such increased selectivity derives into cleaner and higher-quality spectra than conventional HRMS spectra (Dodds and Baker, 2019).

The additional separation accomplished in the mobility device also opens the possibility to resolve coeluting isomeric or isobaric compounds based on inherent structural differences in mobility (Dodds and Baker, 2019; May et al., 2020; Wu et al., 2020). Different studies have been conducted to evaluate the potential of IMS-HRMS to separate and/or differentiate isomeric substances such as carbohydrates (Hofmann and Pagel, 2017), lipids (Zheng et al., 2018b), natural product-based metabolites (Schrimpe-Rutledge et al., 2018), polycyclic aromatic hydrocarbons (Knudsen et al., 2021), polyethylene glycols (Austin et al., 2021), steroids (Rister and Dodds, 2020a) and perfluoroalkyl substances (Ahmed et al., 2019). However, at this moment, the major limitation for an accurate and resolutive separation of coeluting isomers by IMS is the resolving power of the commercially available IMS-HRMS instruments (Eldrid and Thalassinos, 2020; Kaufmann et al., 2020; Wu et al., 2020). Future improvements

in both hardware and software of the instruments will permit to increase the capabilities for isomer resolution.

From a different perspective, the calculation of collision cross section (CCS) values derived from measured DT for each ionized molecule renders an additional identification parameter in conjunction with RT, accurate mass and fragment ions (D'Atri et al., 2018; Dodds and Baker, 2019; Morrison and Clowers, 2019). One of the main benefits of CCS measurement as an extra identification point relies on the evidence that the presence of matrix does not affect the accuracy, robustness and precision of empirical DT measurements (Goscinny et al., 2019; Kaufmann et al., 2020). Therefore, its utilization with confirmation purposes in screening strategies might help to amend RT shifts because of matrix-endogenous compounds.

The implementation of CCS values as an identification parameter has been explored in the literature (Bauer et al., 2018; Goscinny et al., 2019, 2015; Lian et al., 2018; Regueiro et al., 2016; Tejada-Casado et al., 2018). However, only few studies have clearly proposed criteria for confirmation of compound identity considering also IMS as an additional technique (Monge et al., 2019; Nuñez et al., 2019). Monge *et al.* (Monge et al., 2019) presented a scoring system for metabolite identification in untargeted metabolomics as a follow-up for previously reported confidence levels of Sumner *et al.* (Sumner et al., 2007) by the combination of chromatography, MS, IMS and nuclear magnetic resonance. Additionally, Nuñez and coauthors (Nuñez et al., 2019) proposed an automated scoring engine for IMS-HRMS data processing by comparing empirical MS and IMS data with *in silico* libraries. However, neither the chromatographic separation nor mass fragmentation was considered in the latter, which may increase the reporting of false positives. Yet, none of these publications established the minimum requirements for compound identification and, therefore, the implementation of the criteria therein proposed can be different between studies.

In order to facilitate and homogenize the implementation of CCS as an additional identification parameter, the development of CCS databases is pivotal. Due to the

fact that IMS-HRMS for small molecule identification is still in its infancy (Eldrid and Thalassinos, 2020), only few studies have been devoted to create, curate and release databases with empirical CCS data for OMPs (May et al., 2017; Picache et al., 2019; Ross et al., 2020; Zhou et al., 2020). Yet, none of these publications compels relevant information for analytical chemists such as RT, accurate mass for (de)protonated molecules and fragment ions as well as CCS for different adducts. The availability of this type of information will revolutionize the implementation of CCS data for compound identification, especially for those cases in which no reference standard is available, and therefore, third-party data is essential to gain more confidence in suspect findings.

Despite the abovementioned benefits that IMS-HRMS can bring into screening strategies, there is still little knowledge about particular improvements that can be brought to environmental analyses. The utilization of IMS-HRMS instruments for analysis of OMPs in environmental matrices is still limited to a selected group of chemicals such polyfluoroalkyl substances (Dodds et al., 2020; Yukioka et al., 2021), pesticides (Chen et al., 2019), combustion by-products (Zheng et al., 2018a), fatty acids (Mashayekhy Rad et al., 2018), or the screening of organic micropollutants in aquatic environmental samples (Hinnenkamp et al., 2019; Stephan et al., 2016).

2.2. Scientific articles

In this thesis, two different studies have been conducted towards the acceptance of IMS in this scientific field and the application of IMS-HRMS in target, suspect and non-target screening strategies for environmental analyses.

The first work (**scientific article 1**) covers the development, curation and publication of a CCS library for target IMS-HRMS analyses. The database developed contains information about 556 OMPs acquired in both positive and negative ionization modes using electrospray ionization. RT, CCS and accurate mass for precursor and product ions for 970 adducts were included. Additionally, **scientific article 1** presents the first publication including ion

mobility data in widely adopted confidence levels for identification in environmental analyses (Schymanski et al., 2014a).

In addition to the research presented in **scientific article 1**, the second work included in this chapter (**scientific article 2**) depicts the role, potential and main benefits gathered through the utilization of IMS-HRMS instruments for environmental screening. Mass spectral cleaning, CCS robustness measurement and isomeric resolution are discussed by means of illustrative examples from the application of the database and confidence levels previously developed. Also, key aspects of IMS-HRMS, with emphasis on when and with which purpose IMS can be of help in the screening workflow, are examined in **scientific article 2**. Moreover, the effect of including IMS data within the identification criteria for target and suspect compounds is studied by means of the evaluation of the amount of false positive/negative identifications.

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

- *Environmental Science & Technology 54 (2020) 15120-15131*
- *Chemosphere 280 (2021) 130799*

Chapter 2.2.1. Scientific Article 1

IMPROVING TARGET AND SUSPECT SCREENING HIGH-RESOLUTION MASS SPECTROMETRY WORKFLOWS IN ENVIRONMENTAL ANALYSIS BY ION MOBILITY SEPARATION

Alberto Celma, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma

Environmental Science & Technology 54 (2020) 15120-15131

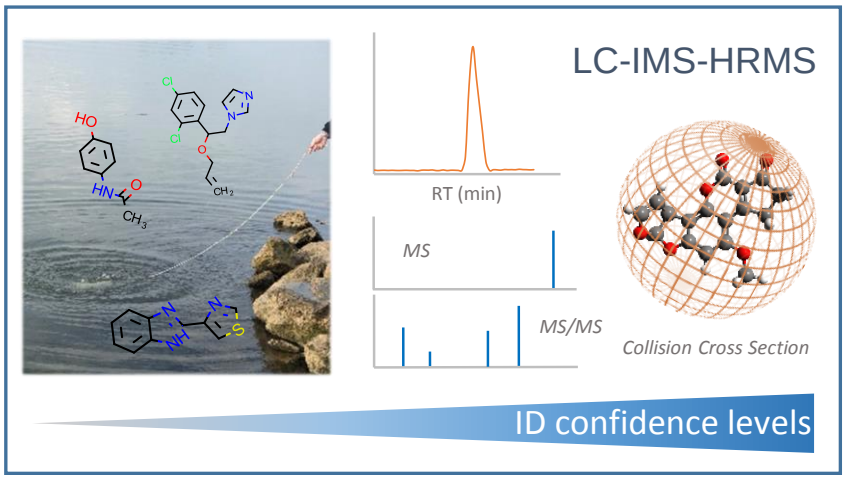
Environmental Science & Technology

pubs.acs.org/est Article

Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in Environmental Analysis by Ion Mobility Separation

Alberto Celma, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández, and Lubertus Bijlsma*

Graphical Abstract



Abstract

Currently, the most powerful approach to monitor organic micropollutants (OMPs) in environmental samples is the combination of target, suspect, and nontarget screening strategies using high-resolution mass spectrometry (HRMS). However, the high complexity of sample matrices and the huge number of OMPs potentially present in samples at low concentrations pose an analytical challenge. Ion mobility separation (IMS) combined with HRMS instruments (IMS–HRMS) introduces an additional analytical dimension, providing extra information, which facilitates the identification of OMPs. The collision cross-section (CCS) value provided by IMS is unaffected by the matrix or chromatographic separation. Consequently, the creation of CCS databases and

the inclusion of ion mobility within identification criteria are of high interest for an enhanced and robust screening strategy. In this work, a CCS library for IMS–HRMS, which is online and freely available, was developed for 556 OMPs in both positive and negative ionization modes using electrospray ionization. The inclusion of ion mobility data in widely adopted confidence levels for identification in environmental reporting is discussed. Illustrative examples of OMPs found in environmental samples are presented to highlight the potential of IMS–HRMS and to demonstrate the additional value of CCS data in various screening strategies.

1. Introduction

High-resolution mass spectrometry (HRMS) offers a powerful and suitable alternative to former targeted screening methods using low-resolution MS [1–5]. The high mass accuracy and resolution, together with the extensive variety of available acquisition modes for a wide mass-to-charge range (*m/z* 50–1000), make HRMS the technique of choice for wide-scope screening of thousands of organic micropollutants (OMPs) and their transformation products in aquatic matrices such as surface water or wastewater [6–9].

Hybrid HRMS mass analyzers, such as quadrupole-time-of-flight (QTOF), offer the possibility of sequentially acquiring information about the ionized molecule and fragment ions, which vastly increases the identification potential of the screening strategy without significantly compromising the sensitivity of the analysis. However, when data-independent acquisition modes are used, fragmentation occurs not only for the compound of interest but also for other co-eluting compounds, and therefore, fragments of multiple precursor ions can contribute to the fragmentation spectrum [6]. Particularly in complex matrices, interferences may be present because of fragment ions from precursors other than the one of interest. As a result, the possibility of misidentification increases. The large amount of data generated, the extensive databases used, and the untargeted acquisition mode applied require meticulous strategies for the identification of compounds in the results obtained. The use of retention time (RT) and mass accuracy tolerance alone during screening analyses can lead to a notable number of false-positive findings [10,11]. To address this, different identification levels have been proposed in the scientific literature, which depend on the information obtained by HRMS analysis [12–18]. The five-level classification, from the most confident scenario (level 1, confirmed structure by a reference standard) to the most uncertain scenario (level 5, exact mass of interest) proposed by Schymanski *et al.* [13], is currently widely used in the environmental literature. While discussions are ongoing for a revised set of identification levels, especially in the metabolomics community, these have not yet achieved community consensus.

The coupling of ion mobility separation (IMS) to HRMS instruments (IMS–HRMS) has promising applications for both targeted and untargeted screening. Briefly, IMS separates ions depending on their size, shape, and charge in a gas phase, usually nitrogen (N_2) or helium (He), in the presence of an electric field [19]. Owing to their different mobilities through the drift cell, IMS enables, in theory, the separation of isobaric or isomeric compounds that could not be previously resolved using liquid chromatography (LC) and/or HRMS [6,19–21]. The time needed by an ion to travel through the mobility separation device, the drift time (DT), is used for the determination of the collision cross section (CCS) of this particular ion based on the measurement of calibrating standards with already established CCS values for travelling wave IMS or trapped IMS instruments or based on the application of Mason–Schamp equation for drift-tube IMS instruments [22]. While the measured DT is not comparable between different instruments [19], CCS is an instrument-independent value that allows for the comparison of CCS libraries with the actual measurement of a candidate in a sample even between different commercially available IMS–HRMS instruments [23]. In light of this, some publications dealing with the creation or use of CCS libraries for hundreds of compounds of different families have been published [24–29]. However, only very few studies have considered the inclusion of ion mobility data into the identification criteria [6,30–36]. Nuñez *et al.* [30] presented an automated scoring engine for the processing of IMS–HRMS data by comparing empirical mass spectrometric and ion mobility data with *in silico* libraries. However, neither chromatographic separation nor mass fragmentation was considered, which may increase the occurrence of false positives. The study conducted by Monge *et al.* [31] proposed a scoring system for the identification of metabolites in untargeted metabolomics as an update for previously reported confidence levels in the study by Sumner *et al.* [18] through the combination of chromatography, MS, IMS, and nuclear magnetic resonance. However, these publications did not establish the minimum requirements for compound identification.

The aim of this work was *(i)* to develop an extensive database of CCS values for hundreds of OMPs in both positive and negative ionization modes, *(ii)* to

incorporate ion mobility information into a wide community-adopted confidence levels for nontarget and suspect screening strategies, and *(iii)* to demonstrate the improved utility of IMS–HRMS in screening of OMPs in environmental samples via illustrative examples gathered in different research projects. The information provided in this work will be of interest in the near future, as it is expected that ion mobility will be incorporated as a complementary criterion for reliable identification in different areas of analytical research.

2. Materials and Methods

2.1. Chemicals and Materials

A total of 556 reference standards comprising illicit drugs, hormones, mycotoxins, new psychoactive substances, pesticides, and pharmaceuticals were injected for the development of a CCS library and the subsequent application of the library to screening analyses. **Table S1** of the **Supporting Information** shows the complete set of compounds used in the study with their SMILES (simplified molecular-input line-entry system) representation, structure, and measured CCS data. The database is also available on the NORMAN Suspect List Exchange website [37], and the Zenodo online repository [38] and the CCS values have been integrated into PubChem [39]. JChem for Office (version 19.9.0.467) in Excel (from ChemAxon, www.chemaxon.com) was used for chemical parameters and structure calculation [40].

2.2. Instrumentation

Analyses were performed with a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) connected to a VION IMS-QTOF mass spectrometer, using an electrospray ionization interface operated in both positive and negative ionization modes.

The chromatographic column used was a CORTECS C18 2.1 × 100 mm, 2.7 µm fused core column (Waters) at a flow rate of 300 µL min^{-1}. Gradient elution was performed using H_2O (A) and MeOH (B) as mobile phases, both with 0.01% formic acid. The initial percentage of B was 10%, which immediately linearly increased to 90% over 14 min, followed by a 2 min isocratic period, and then returned to initial conditions (at 16.1 min) with a 2 min equilibration of the column. The total run time was 18 min. The injection volume was 5 µL.

A capillary voltage of 0.8 kV and cone voltage of 40 V were used. The desolvation temperature was set to 550 °C, and the source temperature was set to 120 °C. Nitrogen was used as the drying gas and nebulizing gas. The cone gas flow was 250 L h^{-1}, and the desolvation gas flow was 1000 L h^{-1}. The column temperature was set to 40 °C, and the sample temperature was set to 10 °C. MS data were

acquired using the VION in HDMS^E mode, over the range *m/z* 50–1000, with N_2 as the drift gas, an IMS wave velocity of 250 m s^{-1}, and wave height ramp of 20–50 V. Leucine enkephalin (*m/z* 556.27658 and *m/z* 554.26202) was used for mass correction in positive and negative ionization modes, respectively. Two independent scans with different collision energies were acquired during the run: a collision energy of 6 eV for low energy (LE) and a ramp of 28–56 eV for high energy (HE). A scan time of 0.3 s was set in both LE and HE functions. Nitrogen (≥99.999%) was used as a collision-induced dissociation gas. All data were examined using an in-house built accurate mass screening workflow within the UNIFI platform (version 1.8.2) from Waters Corporation.

2.3. CCS Library

The whole set of reference standards was divided into different mixtures of up to 20 compounds depending on substance classes, based on previous knowledge about chromatographic separation, and avoiding the presence of isobaric and isomeric compounds in the same mixture.

To obtain an accurate CCS value for each compound, the following workflow was used. Prior to the standard injection, the instrument was calibrated both for *m/z* measurements and CCS calculation, following the manufacturer's instructions. Then, a "system suitability test" (SST) containing nine compounds was injected ten times to check the accuracy of the instrument measurements. **Table S2** shows the compounds included in the SST, their molecular formula, SMILES, and *m/z* and CCS values. Expected CCS values were provided by the manufacturer; data measurements were performed in triplicate at three different pressures of N_2 with a minimum of eight different voltage gradients [relative standard deviation (RSD) was typically <0.3%] using a modified Synapt G2-Si (linear drift tube in place of the standard travelling wave cell). Then, in this study, reference standard mixtures at 1, 10, and 100 µg L^{-1} were injected in triplicates. After every mixture sequence (*i.e.,* all injections of the three concentration levels per mix), the SST was performed for temporal evolution and the continuous control of the stability of the measurement. At the end of the sequence, the SST was performed again (*n*=10). For the data to be considered acceptable, mass accuracy and CCS

error (percentage deviation from the expected value) for the start, end, and interspersed SST injections had to be within an acceptable tolerance (3 ppm in mass accuracy and 2% deviation in CCS). **Figure S1** shows the temporal evolution of mass and CCS accuracy across a representative injection run of standards during the building of the CCS library with interspersed SST in the positive ionization mode. As expected, the empirical CCS deviation was below ±2% deviation (mostly <1%), ensuring a good robustness of the CCS measurement.

The actual value of CCS for a compound was established by averaging the nine values obtained at the three concentrations tested. In the cases where no signal was observed in the lower concentration level, the CCS value was established by averaging the data for the other concentration levels.

3. Results and discussion

3.1. CCS Library

A library containing CCS information of a total of 970 different adducts corresponding to 556 compounds (209 pesticides, 170 pharmaceuticals, 128 illicit drugs and new psychoactive substances, and 49 hormones and mycotoxins) was built to enhance target workflows with IMS. The library contains 472 protonated adducts ($[M+H]^+$), 248 sodium adducts ($[M+Na]^+$), 26 water loss in-source fragments ($[M+H-H_2O]^+$), 9 ammonia loss in-source fragments ($[M+H-NH_3]^+$), 162 deprotonated adducts ($[M-H]^-$), 25 chlorinated adducts ($[M+Cl]^-$), and 31 formate adducts ($[M+HCOO]^-$). The complete library is available in **Table S1** of the **Supporting Information** and is also publicly available on the NORMAN Suspect List Exchange website [37], Zenodo online repository [38], and on PubChem [39].

As previously mentioned, the CCS for each adduct was obtained as an average value of the replicates injected at three different concentration levels. In general, the RSD observed between replicates was 0.1–0.3%, and no trend was observed in the CCS measurement precision depending on the concentration of the reference standard. As an example of the main trend, **Figure S2** shows the RSD in the measurement of CCS values for a set of 46 pesticides. The robustness of CCS measurements across injections supports the use of ion mobility as a powerful and promising tool for the improved identification of candidates.

In general, the CCS value of a certain adduct is strongly related to the molecular mass, such that different adducts of the same molecule generally result in different CCS values because of the difference (mainly in size) of the ion incorporated in or removed from the structure [41] (**Figure S3**). However, the nonperfect linear correlation between CCS and molecular mass (see **Section S1**) highlights that CCS values are also affected by other molecular parameters, such as the chemical backbone and ionization site, or how the molecule can rearrange its structure to stabilize the electric charge. This is particularly the case for X-ray agents —ioversol, iopromide, iomeprol, and iopamidol— which with a molecular mass of approximately 800 Da yield an unexpected low CCS value because of the

intrinsic characteristics of the chemical backbone and substituents (**Figure S4**). Furthermore, it is noteworthy that among the complete set of 556 reference standards analyzed, only protomers were observed for the quinolone antibiotics sarafloxacin (I: 187.09 $Å^2$ and II: 202.00 $Å^2$), ciprofloxacin (I: 175.38 $Å^2$ and II: 188.89 $Å^2$), and norfloxacin (I: 171.88 $Å^2$ and II: 187.60 $Å^2$). In these particular cases, protonation on the cyclic ketone or the piperazine moiety [21] (**Figure S5**) resulted in different conformational changes, being distinct enough to be resolved by IMS. Consequently, these protomers could be qualitatively identified in real samples using IMS–HRMS without the need to consider abundances within the identification strategy.

A detailed and comprehensive discussion concerning the general trends observed for CCS values and these particular cases can be found in the **Supporting Information** (**Section S1** and **Figures S3–S6**).

3.2. Identification Levels for IMS–HRMS Screening Strategies

Having well-defined criteria accepted by the scientific community for the identification of candidates in screening strategies is pivotal for an accurate dissemination of results and the comparison of results with other studies. For that purpose, Schymanski *et al.* [13] proposed a five-level criteria for the identification of small molecules using chromatographic separation coupled to HRMS. This classification also included cases in which the solely available information was their molecular formula or exact mass (level 4 —unequivocal molecular formula— and level 5 —exact mass of interest). At these levels, insufficient information is available to propose tentative candidates. However, the data available for level 3 —tentative candidate(s)— allow for the proposition of more than one chemical structure (*e.g.,* positional isomers). Candidate structures elucidated by *in silico* fragmentation tools are usually most appropriately classified as level 3 features. Level 2 —probable structure— is related to candidates that could unambiguously be assigned to a certain chemical structure based on the scientific literature, mass spectral libraries, or diagnostic evidence. Finally, level 1 —confirmed structure— represents the ideal situation, where chromatographic and mass spectrometric evidence are confirmed with a

reference standard. These criteria have been widely adopted by environmental researchers [3,42–44]. Even though the fragmentation information gathered with HRMS instruments often determines the potential for identification of candidates, the utilization of additional orthogonal methods is recommended [13,45]. In this sense, the incorporation of IMS–HRMS in screening strategies permits us to gain even more confidence in the identification and adds an extra dimension to further improve screening analyses [46]. The inclusion of IMS may also help to discriminate between isomeric level 3 candidates and move one of them up to level 2. In this work, recommendations are given on how to apply the five-level criteria from the study by Schymanski *et al.* [13] for users of state-of-the-art IMS–HRMS instruments. The analytical experience gathered during the CCS library building has been taken into account in proposing these criteria. Classification is intended to enhance these widely adopted criteria, suggest how to apply them to IMS–HRMS measurements, as well as to contribute to the community discussion on how to incorporate multiple lines of evidence into identification confidence schemes.

Figure 1 shows different levels of confidence proposed in this work for the identification of a compound using LC–IMS–HRMS based on chromatographic, ion mobility, and mass spectrometric parameters. Typically, the accuracy of empirical data for mass spectrometric measurements is established at a maximum deviation of 5 ppm (or 2 mDa) from the theoretical *m/z*, as well as compliance with the expected isotopic pattern [45]. However, as most HRMS instruments can provide higher levels of accuracy, the threshold for deviation in mass spectrometric measurements could nowadays be adjusted to 3 ppm. The criterion for RT is less harmonized among the scientific community, and it is surely more debatable. In this work, a maximum RT deviation of ±0.1 min from that of the standard is proposed to be in agreement with the SANTE 2017 guideline [45], implying that both the sample and reference standard are run under the same chromatographic conditions. However, the SANTE guideline is applied for food analysis and not for environmental analysis. As such, the maximum deviation is an indicative value and should be adapted depending on the particular conditions of the analysis. The results obtained and the examples

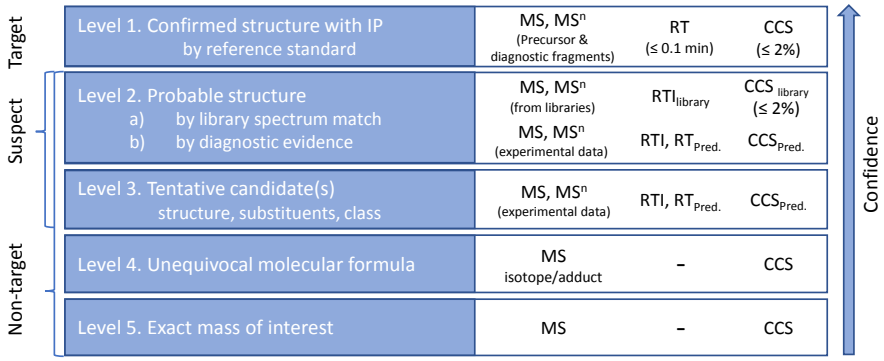


Figure 1. Different confidence levels established in the identification of a compound applying ion mobility HRMS target, suspect and nontarget screening workflows based on the levels provided by Schymanski *et al.* [13]. MS refers to accurate mass of the precursor ion, MS^n to accurate mass of the fragment ions, RT is the retention time, RTI refers to retention time indexing systems, CCS means collision cross-section, and the sub index Pred. indicates that the value is in accordance with predictive models applied.

presented in this study may open the dialogue to develop more applicable criteria for environmental studies, where matrix effects can potentially lead to high deviations. In the case of CCS, there are no regulatory guidelines yet, and therefore, there is still no agreement on which is the maximum threshold permitted for CCS deviation. Based on the experience gathered during the development of the CCS library included in this study, together with the background knowledge acquired during screening campaigns using IMS–HRMS, we propose a maximum deviation of 2% for CCS values. Depending on the availability of reference standards, in addition to the accuracy of the acquired empirical data, the level classification previously proposed by Schymanski *et al.* [13] is updated for IMS–HRMS users as follows:

Level 5 *—exact mass of interest—* represents the level where least information about the candidate is available. However, the exact mass together with its specific CCS value is considered relevant for the study and worth being monitored in future campaigns.

Level 4 *—unequivocal molecular formula—* encompasses the cases where a molecular formula can be assigned. MS, RT and CCS information alone, without

fragmentation information, is commonly not enough to propose a potential structure, and therefore, RT and CCS data measured typically do not provide sufficient additional information for identification.

Level 3 —*tentative candidate(s*)— comprises the cases where different chemical structures are compatible with the empirical RT, CCS, and MS data, but not enough information is available to distinguish which one is the most likely. In these cases, empirical information about the chromatography, ion mobility, and MS behavior of the candidates could be compared with predicted parameters. The predictions about the value for RT, CCS, or mass fragmentation can give extra confidence to the proposed tentative candidates [47–54] or help prioritize potential candidates [46]. Despite the additional value of such tools, the predicted values should be considered as an orientation. Hence, rejecting candidate structures solely because of a disagreement between empirical and predicted values is not recommended. The utilization of RT indexing (RTI) strategies to compare the empirical data with online available databases can also provide extra confidence in the tentative identification of candidates [11,55].

Level 2 —*probable structure*— indicates that an exact structure could be proposed based on experimental evidence. This level can be divided into two sublevels. ***Level 2a*** —*probable structure by library match*— comprises those cases when the structure of the compound is proposed based on the agreement between experimental data and literature or available libraries for both HRMS and CCS. The high robustness of the CCS measurement between different instruments permits the utilization of home-made or third-party CCS libraries to compare with experimental data, reaching a high level of confidence in the identification. ***Level 2b*** —*probable structure by diagnostic evidence*— makes use of the available data to unambiguously propose a structure in the case that no other candidate fits the empirical evidence. The slight difference between level 2b and level 3 is the fact that, in level 2b, only one structure satisfies the experimental evidence (and all other candidates can be eliminated), while in level 3, there is not enough evidence to distinguish between more than one candidate structures. Level 2b identifications are generally quite rare and often require experimental context (*e.g.,* transformation experiments where the

parent is known). For both levels 2a and 2b, a reference standard is required for the final confirmation of the structure to achieve the highest confidence (level 1).

Level 1 *—confirmed structure—* is the ideal situation, where the empirical data fully agree with that of a reference standard in terms of MS, fragmentation, RT, and CCS. This is the case where the highest confidence in the identification is obtained with HRMS. For a proper level 1 identification, all orthogonal techniques (MS, fragmentation, RT, and CCS) should be in accordance with those of the reference standard. However, the comparison of the reference standard information to empirical data from samples can result in different sublevels of identification confidence. Hence, the combined adoption of an identification point (IP) scoring system to address this often to be a challenging task is proposed in agreement with the Commission Decision 2002/657/EC [56] and recently reported IP proposals [42,57]. Briefly:

- Empirical MS information matches the reference standard, 1 IP
- Empirical RT information matches the reference standard, 1 IP
- Empirical CCS information matches the reference standard, 1.5 IP
- Two or more matching HRMS fragments, 2.5 IP
- Minimum IP for level 1 identification with CCS, 5 IP

Although the ideal situation should yield a maximum of 6 IP (1 for MS, 1 for RT, 2.5 for HRMS fragmentation, and 1.5 for CCS), a minimum value of 5 IP should be considered sufficient for the confirmation of the identity. While some studies have proposed different criteria for the identification of compounds [13,42,45,56,57], very few consider the likely case in which any of the parameters measured (RT, CCS, and mass spectrometric data) fails to meet the requirements. In such cases, establishing the level of confidence of the identification is not a straightforward decision and usually further investigation is required to accurately report the detection. Mass spectrometric data can be affected by several factors, and therefore, when the mass accuracy is barely higher than the established threshold, different actions can be followed. The immediate verification should be the instrument performance by checking the mass accuracy with a set of reference standards injected alongside the sample

injection run as quality controls. In addition, spectral interferences can affect the mass accuracy, which can be improved by a reinjection of the sample with enhanced resolution (which is often not available for many instruments). The dependence of mass error on the signal intensity is also important. The lower the number of ions measured, the higher the mass error; therefore, low abundant fragments often show higher mass errors [58]. The same applies for high intensity ions, which can distort mass accuracy because of detector saturation.

On another point, either an RT error slightly higher than 0.1 min or ΔCCS slightly greater than 2% would require the fortification of the original sample with the candidate compound and/or modification in the chromatographic conditions to fully confirm its identity. However, in our own experience, the chance of having deviations greater than 2% in the CCS is low because of the robustness of the CCS measurements. Therefore, not all the parameter deviations should be weighted uniformly because RT is more prone to be shifted by the sample matrix [11]. Consequently, variation in RT slightly questions the identification of a candidate that perfectly matches the reference standard for HRMS data and CCS. On the contrary, CCS deviation higher than 2% strongly questions the identification. In this sense, the minimum requirement for identity confirmation as level 1 is established at 5 IP, which already considers the possibility of deviations in RT but needs an agreement of CCS. For those particular cases, when empirical data do not completely fit the reference standard, reporting the candidate at the corresponding level with a reduced score (<5 IP), accompanied by clarification on the parameter failing in the requirements, is proposed in order to comprehensively report the data (*e.g.,* highlighted with an asterisk as *level x**). Obviously, the fact that one parameter (commonly RT and mass error) is slightly out of tolerance (typically 0.1 min and 5 ppm, respectively) would reduce the confidence, but might not be as crucial as other important parameters, such as CCS deviation or the presence of fragment ions in agreement with experimental data or spectral libraries.

3.3. Application to Environmental Water Samples

The application of efficient strategies for the wide-scope screening of OMPs in environmental samples has become essential. While strategies involving HRMS may lead to misidentifications in some cases [11,35,59–63], IMS–HRMS instruments provide an extra identification parameter that improves the performance and helps to reduce the number of false positives/negatives [10]. In this section, we highlight different identification scenarios using the developed CCS library to show the potential of IMS–HRMS in environmental analysis. It summarizes some of the experience gathered through the utilization of IMS–HRMS in different research studies.

Figure 2 shows the confirmation at level 1 of 4-acetamidoantipyrin in surface water from a nature reserve in Spain after preconcentrating the sample using solid-phase extraction. Despite being a protected area, the sampling location was contaminated through the introduction of the effluent stream of an urban wastewater treatment plant. The presence of this human metabolite of metamizole can be attributed to an inefficient removal during wastewater treatment. The entry in the CCS library for the reference standard of 4-acetamidoantipyrin showed a RT of 3.01 min with a CCS value of 154.06 $Å^2$ for the protonated molecule and HE fragment ions with *m/z* 228.1132 and *m/z* 104.0495. The candidate observed in the surface water sample eluted at 3.09 min (+0.08 min of deviation), and both the protonated molecule and the HE fragments were observed at their *m/z* (mass error < 3 ppm). In addition, the experimental CCS for the candidate was 154.08 $Å^2$, which only deviates by +0.01% from the standard. In the light of the full agreement of all these measurements and using the criteria previously proposed, the identification of this candidate as 4-acetamidoantipyrin was confirmed as level 1 with 6 IP (MS + RT + >2 HRMS fragments + CCS).

As stated above, in environmental samples, the matrix composition can strongly influence compound retention and, therefore, the RT for most of the analytes [11]. This fact may lead to a notable increase in the number of misidentifications because of significant RT deviation between the standard and sample.

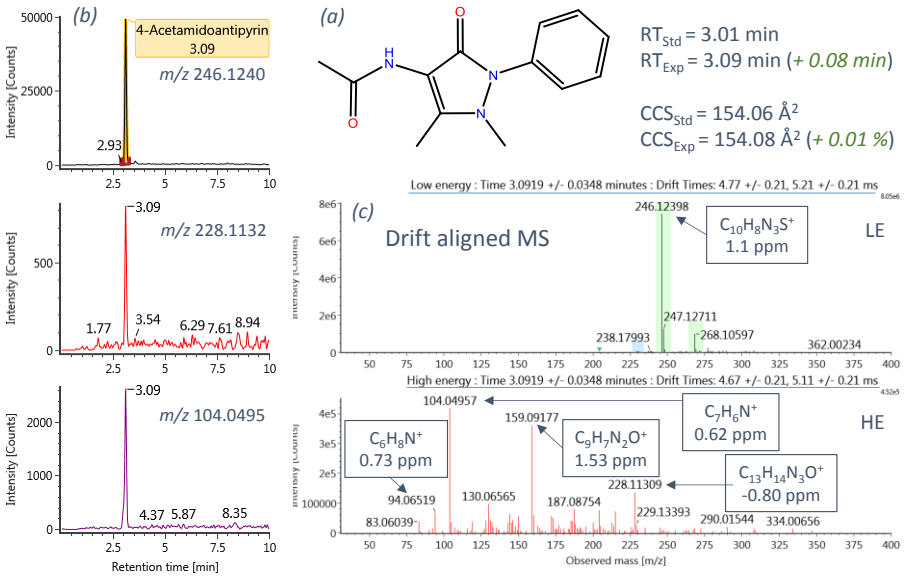


Figure 2. Identification at level 1 of 4-acetamidoantipyrin in a surface water sample. *(a)* Structure, RT and CCS comparison of experimental and standard data, *(b)* extracted ion chromatograms for $[M + H]^+$ ion (*m/z* 246.1240) and two characteristic fragments (*m/z* 228.1132 and *m/z* 104.0495) and *(c)* DT aligned MS data along with the empirical mass error of the corresponding fragment ions observed.

Nevertheless, the excellent reproducibility observed for CCS values, and the fact that this parameter is not affected by matrix composition and provides extra identification power, which is especially useful for compounds partially out of the confirmation criteria. As an illustrative example, **Figure 3** shows the detection of thiabendazole, a fungicide used to control fungal diseases in fruits and vegetables, in the mouth of a Spanish river in the Mediterranean basin identified at level 1* (*i.e.*, RT deviation beyond limits). The RT for the thiabendazole reference standard was 3.27 min with a CCS value of 137.44 Å². However, the RT in the sample was 3.51 min and seemed notably affected by matrix composition, with a deviation of +0.24 min. The RT difference between standard and sample is far in excess of the typical criterion established for confirmation (±0.1 min) (**Figure 1**) not earning, in consequence, the 1 IP for RT agreement. On the contrary, ion mobility was not affected by the matrix and resulted in a CCS value of 137.27 Å², which only deviated −0.12% from the

standard. In addition, the protonated molecule and three fragments were observed with mass errors below 3 ppm. Under these conditions, the identity of this compound as thiabendazole could be confirmed at level 1* with 5 IP (MS + >2 HRMS fragments + CCS). This example illustrates that RT affected by matrix composition may hamper the confirmation process in wide-scope screening, while the application of CCS provides the extra value needed for confirmation. In cases in which the RT notably deviates from the standard, some guidelines recommend to spike the sample with the candidate standard to confirm the identity of the compound [45]. However, the additional confidence gathered with the CCS measurement in a single-injection reduces time and costs of spiking and reinjecting the sample, as two separate pieces of evidence already exist (MS + >2 HRMS fragments + CCS). This is of special interest in environmental screening strategies, where ion mobility can be included as an additional criterion for reliable identification in forthcoming guidelines in different fields of analytical research.

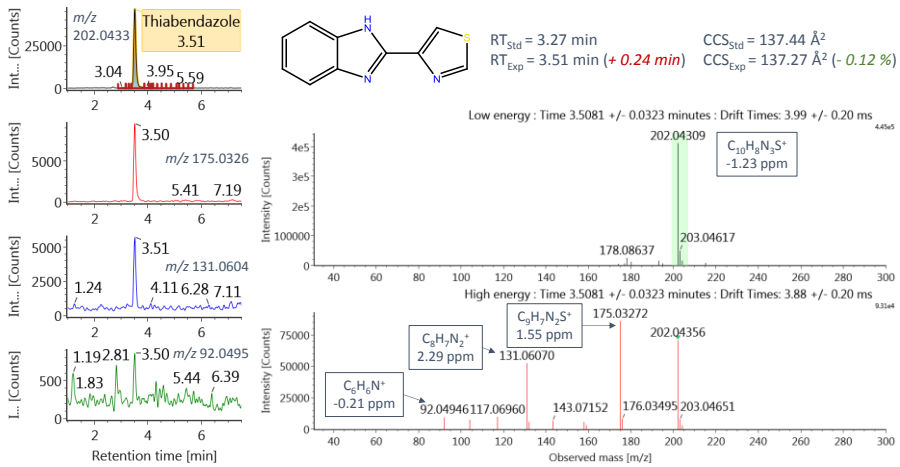


Figure 3. Identification as level 1* of the fungicide thiabendazole in a Spanish river mouth including structure and CCS comparison of experimental and expected data (right top panel), extracted ion chromatograms for $[M+H]^+$ ion (*m/z* 202.0433) and 3 representative fragments (*m/z* 175.0326, *m/z* 131.0604, *m/z* 92.0495) (left panel) and DT aligned MS data with the empirical mass error of the fragment ions observed (right-bottom panel).

Moreover, the robustness of CCS measurements allows this parameter to be used also as an extra point of confidence when the reference standard is not available. Prediction tools can offer an estimation of the CCS value that can easily be compared to the measured value of the tentatively identified compound [49,51,54]. This is the case of the tentative identification of valifenalate in spinach samples reported by Bijlsma *et al.* [49], who found a potential positive with an experimental CCS of 196.97 $Å^2$, although no reference standard was available for confirmation. By means of a predictive model developed using artificial neural networks, the authors were able to predict CCS values for small molecules. The predicted CCS for valifenalate was 194.34 $Å^2$, which deviated only 1.4% from the experimental value, resulting in higher confidence in the tentative identification. Similarly, in the present work, a suspect screening of pesticides in surface water revealed a potential positive of tricyclazole, commonly used for the control of *Magnaporthe grisea* fungi during rice blast. The candidate peak ($[M+H]^+$; *m/z* 190.04354) showed a RT of 5.74 min with the fragment ions *m/z* 163.03251, *m/z* 136.02158, *m/z* 109.01057, and *m/z* 92.04961 and a measured CCS of 133.93 $Å^2$ (**Figure 4**). HRMS information contained in the free online-available mass spectral database MassBank of North America [64] included four fragment ions for tricyclazole (*m/z* 163.0333—$C_8H_7N_2S^+$, *m/z* 136.0220—$C_7H_6NS^+$, *m/z* 109.0106—$C_6H_5S^+$, and *m/z* 92.0496—$C_6H_6N^+$), which fully agreed with our experimental data. Additionally, the CCS prediction model developed by Bijlsma *et al.* [49] predicted a CCS value of 136.2626 $Å^2$, with a deviation of +1.74% from the experimental measurement. Although the reference standard should be acquired for the full confirmation of the identity, the agreement of all these parameters gave high confidence to the tentative identification of tricyclazole in the surface water sample, at level 2a. At a later stage, the reference standard was purchased, and it allowed for the identification of tricyclazole at level 1 because full agreement between empirical and reference standard data was achieved (reference standard data: RT 5.78 min, CCS 132.98 $Å^2$, $[M+H]^+$ *m/z* 190.04354—$C_9H_8N_3S^+$ and fragments *m/z* 163.03245—$C_8H_7N_2S^+$, *m/z* 136.02155—$C_7H_6NS^+$, *m/z* 109.01065—$C_6H_5S^+$, and *m/z* 92.04948—$C_6H_6N^+$).

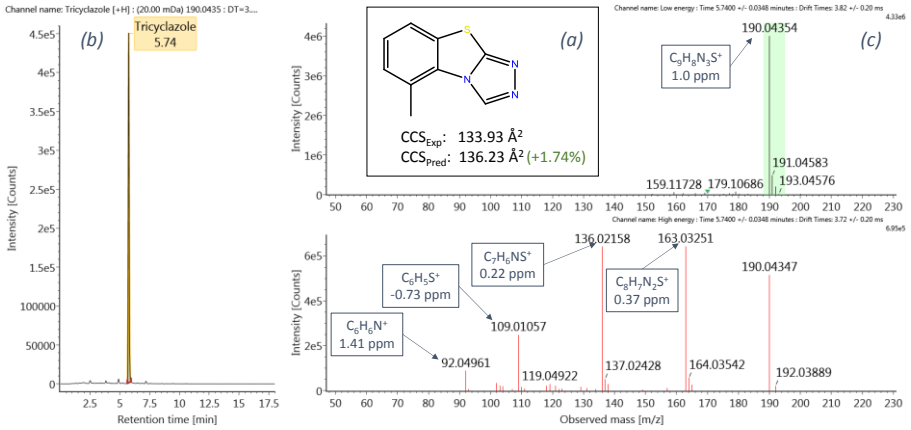


Figure 4. Identification at level 2a of tricyclazole in a surface water. *(a)* Structure and CCS comparison of experimental and predicted data, *(b)* extracted ion chromatogram for $[M+H]^+$ ion (*m/z* 190.0435) of tricyclazole and *(c)* DT aligned MS data along with the empirical mass error of the fragment ions observed.

It is worth emphasizing at this point that the proposed levels of confidence in the identification and the discussion of the examples are both based on the knowledge gathered by the authors through the use of IMS–HRMS in several studies. The expertise of the mass spectrometrist should be the rationale behind the application of the levels of confidence for IMS–HRMS analyses. The results from screening should be deeply reviewed by experienced researchers and data critically discussed if there is a deviation on the criteria (such as mass spectrometric accuracy or RT deviation), avoiding immediate exclusion of potential positives by an automated application of strict criteria. Although the use of mass spectrometric databases and/or predictive models give more confidence into the results, the experience of the analyst is crucial in the elucidation of compounds through the utilization of common mass fragmentation rules [65]. Additionally, the sample origin and its characteristics can be determinant when considering potential candidate structures for the empirical features, and this knowledge can only come up from a human being and not (yet) from an automated processing software.

3.4. Strengths and Limitations of IMS–HRMS

The use of IMS–HRMS for wide-scope screening of OMPs in environmental analyses is a powerful instrument for an enhanced analytical performance. One of the major benefits of ion mobility, which is usually insufficiently acknowledged in the scientific literature, is the simplification of mass spectral interpretation. In addition to separating chromatographically co-eluting ions, ion mobility also filters both LE and HE spectra, removing ions that do not belong to the candidate of interest [6,33]. This includes the removal of other co-eluting compounds that could be producing HE fragments and the reduction of matrix-endogenous interferences, thereby decreasing the number of peaks in a spectrum to be interpreted and thus also the risk of false fragment library matching. As an illustrative example, **Figure 5** shows the comparison of LE and HE spectra of benzoylecgonine, the main metabolite resulting from cocaine use, of a reference standard (**Figure 5a**) and a positive finding in a wastewater sample with the DT aligned (**Figure 5b**) and non-DT aligned spectra (**Figure 5c**). When no IMS is applied (**Figure 5c**), the spectrum is much more populated with ions that do not originate from benzoylecgonine than in the DT aligned spectrum (**Figure 5b**), with a quality comparable to the reference standard spectrum. The fact that IMS–HRMS provides "clean" spectra because of matrix interferences and co-eluting ion separation strongly facilitates the spectral interpretation and identification process in wide-scope screening strategies, especially in comparison with non-ion mobility HRMS instruments [6].

Despite the benefits of IMS–HRMS, some limitations should also be mentioned. The IMS–HRMS instrument used in this study, VION IMS-QTOF mass spectrometer from Waters, has the mobility separation cell located between the ionization source and the mass analyzer. Therefore, ions constantly produced in the ionization source need to be packed in small groups of ions every 14 ms in order to separate them by their mobility. To this aim, a trap is located before the separation cell. Unfortunately, the release process of the trapped ions seemed to cause additional fragmentation in the LE function for labile (de)protonated molecules.

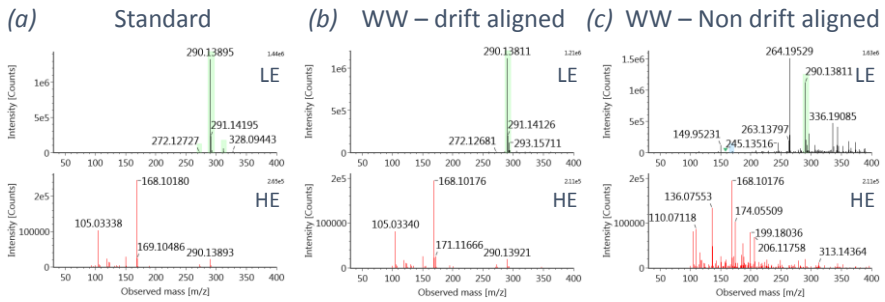


Figure 5. Comparison of HRMS spectra for benzoylecgonine in analytical reference standard solution *(a),* DT aligned data of positive finding in wastewater sample *(b)* and non-DT aligned data of the same positive finding in wastewater *(c).*

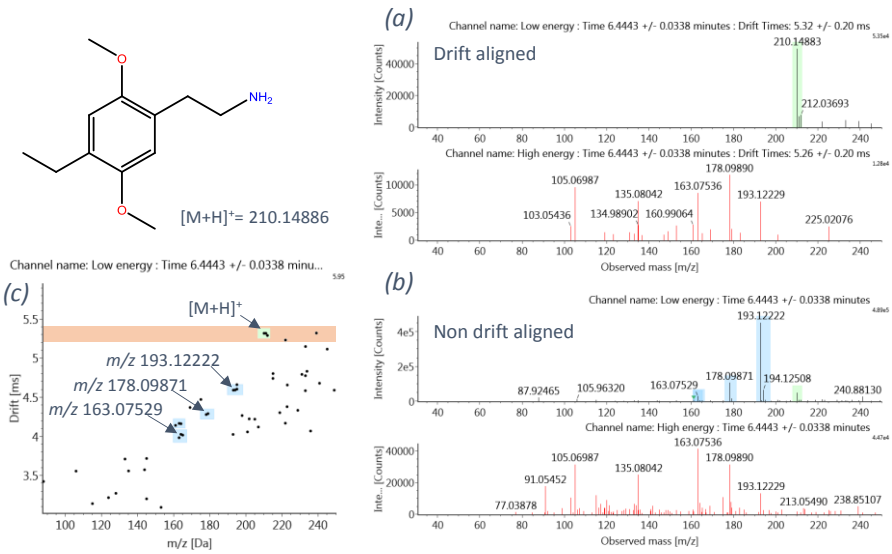


Figure 6. "Pre-mobility" fragmentation of 2C-E resulting in LE fragments with different DT (blue-shadowed points) *(c)* which are omitted in the DT aligned data for protonated adduct (green-shadowed peak) *(a)* but present in the non-DT aligned data (blue-shadowed peaks) *(b).*

As an example, **Figure 6** highlights the LE fragmentation for the new psychoactive substance 2,5-dimethoxy-4-ethylphenethylamine (2C-E). A routine revision of HRMS data in screening analyses is often performed, making use of the aforementioned advantages of IMS–HRMS, and therefore, revising DT aligned MS data. That would be the case of spectra shown in **Figure 6a**, which

apparently is a proper spectrum for a potential positive of 2C-E with a protonated adduct *m/z* 210.14883 in the LE function and significant fragments in the HE function. However, the non-DT aligned MS spectra (**Figure 6b**) show that the most abundant ion does not really correspond to the protonated adduct (*m/z* 210.14886, green shadowed) but to the ammonia loss fragment (*m/z* 193.12222, blue shadowed), followed by other LE fragments such as *m/z* 178.09871 and *m/z* 163.07529. Further investigation revealed that all these ions showed different ion mobilities (different DT) (**Figure 6c**), which confirms that they were produced at some stage before the mobility separator device. The extra fragmentation observed was confirmed to be a "pre-mobility" fragmentation behavior but not an enhanced "in-source" fragmentation because fragmentation did not occur when working in the conventional MS^E mode (*i.e.,* with no mobility separation) (**Figure S7**). This "pre-mobility" fragmentation produced a 10-fold decrease in the intensity of the protonated adduct of 2C-E, which may hamper the discovery of this compound in a real-sample scenario. Therefore, this particular "pre-mobility" fragmentation may have negative consequences in environmental analysis where most of detections and subsequent identifications are based on the presence of the protonated molecules. The reduced intensity of the protonated adduct of the molecule can favor false negative identifications, especially for low abundant and very labile compounds such as some psychoactive substances in wastewater samples. It is noteworthy that this particular example was observed using a VION IMS-QTOF instrument and, therefore, cannot be directly extrapolated to other IMS instruments. However, the nature of IMS separation and the building of mobility devices may possibly lead other manufacturer instruments to suffer from a similar "pre-mobility" phenomenon.

In summary, although the abovementioned limitations have been observed, IMS–HRMS has a strong potential for wide-scope screening of OMPs and notably facilitates screening strategies in highly complex matrices. The much cleaner DT aligned MS spectra enhance the identification process, and the excellent robustness of CCS measurements in different matrices enables CCS prediction tools to help in the tentative identification of candidates when the reference

standard is not available. This enhances the confirmation rate if the reference is eventually acquired for confirmation. Furthermore, freely and/or commercially available CCS libraries, both measured and computational, can be used to facilitate target/suspect screening because of the stability and extra identification power provided by ion mobility when RT shifts are likely to occur.

In this paper, we provided a publicly available data set of 970 CCS values, illustrated the potential of IMS–HRMS, and suggested IMS-based scoring criteria to enhance commonly applied identification reporting levels in environmental analyses. The work was supported by real examples, taking into account the additional value of ion mobility, and demonstrated an improved screening strategy for OMPs in environmental samples based on state-of-the-art IMS–HRMS technologies.

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Supporting information

This section contains information on the compounds used for the "SST", temporal evolution of mass accuracy and CCS accuracy, general trend observed in the ion mobility measurement during the CCS library building process, CCS values of different adducts versus the neutral mass of the molecule, chemical structures of X-ray agents ioversol, iopromide, iomeprol, and iopamidol, different protomers separated by IMS–HRMS, absolute variation of the CCS value observed in molecules showing more than one ionic species, HRMS spectra for 2C-E, and main trends and particularities observed during CCS library development. It also contains the complete database for 970 (de)protonated adducts, sodiated adducts, ammonia loss, water loss, chlorine adducts and formate adducts as a separate Excel file.

Supporting Information to this article can be found, in the online version, at https://pubs.acs.org/doi/10.1021/acs.est.0c05713 and in this chapter after the section "References".

Due to space limitations, Table S1 is not included in this thesis, and can be found in the online version of this article (https://pubs.acs.org/doi/10.1021/acs.est.0c05713), at https://www.norman-network.com/nds/SLE/ (list S61); https://doi.org/10.5281/zenodo.3549476; and https://pubchem.ncbi.nlm.nih.gov/source/23819#data=Annotations.

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Supplementary data

Table S2. SMILES, molecular formula, expected adduct, *m/z* and CCS values for the set of 9 compounds included in the 'System Suitability Test' (SST) mix.

Compound Name	SMILES	Molec. Form.	Adduct	Expected *m/z*	Expected CCS (Å²)
Acetaminophen	CC(=O)Nc1ccc(cc1)O	$C_8H_9NO_2$	-H	150.0651	131.50
			+H	152.0706	130.40
Caffeine*	Cn1cnc2c1c(=O)n(c(=O)n2C)C	$C_8H_{10}N_4O_2$	+H	195.0877	138.20
Leucine Enkephalin	CC(C)C[C@@H](C(=O)O)NC(=O)[C@H](Cc1ccccc1)NC(=O)CNC(=O)CNC(=O)[C@H](Cc2ccc(cc2)O)N	$C_{28}H_{37}N_5O_7$	-H	554.2620	225.40
			+H	556.2766	229.80
Reserpine	COc1ccc2c(c1)[nH]c3c2CCN4[C@@H]3C[C@H]5[C@@H](C4)C[C@H]([C@@H]([C@H]5C(=O)OC)OC)OC(=O)c6cc(c(c(c6)OC)OC)OC	$C_{33}H_{40}N_2O_9$	-H	607.2661	265.20
			+H	609.2807	252.30
Sulfadimethoxine	COc1cc(nc(n1)OC)NS(=O)(=O)c2ccc(cc2)N	$C_{12}H_{14}N_4O_4S$	-H	309.0663	170.10
			+H	311.0809	168.40
Sulfaguanidine	c1cc(ccc1N)S(=O)(=O)NC(=N)N	$C_7H_{10}N_4O_2S$	-H	213.0452	145.20
			+H	215.0597	146.80
Terfenadine*	CC(C)(C)c1ccc(cc1)C(CCCN2CCC(CC2)C(c3ccccc3)(c4ccccc4)O)O	$C_{32}H_{41}NO_2$	+H	472.3210	228.70
Val-tyr-val	CC(C)[C@@H](C(=O)N[C@@H](CC1=CC=C(C=C1)O)C(=O)N[C@@H](C(C)C)C(=O)O)N	$C_{19}H_{29}N_3O_5$	-H	378.2034	192.50
			+H	380.2180	191.70
Verapamil*	CC(C)C(CCCN(C)CCc1ccc(c(c1)OC)OC)(C#N)c2ccc(c(c2)OC)OC	$C_{27}H_{38}N_2O_4$	+H	455.2904	208.80

*Only observed in positive ionization mode.

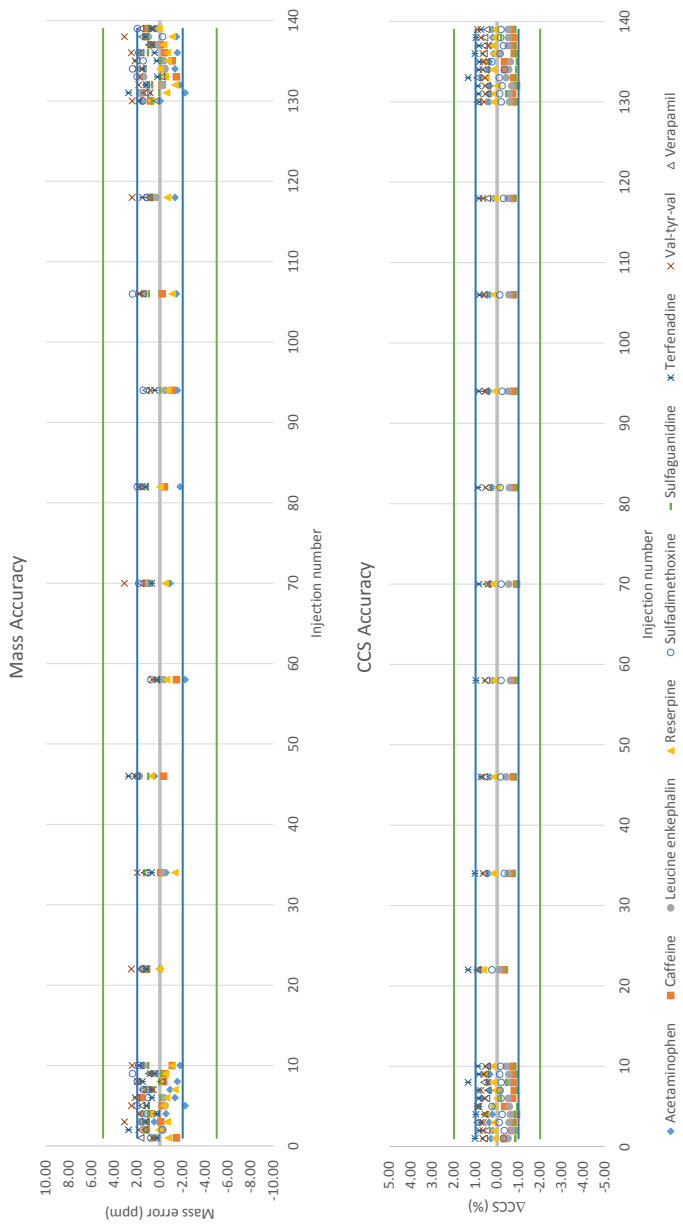

Figure S1. Temporal evolution of mass accuracy (upper) and CCS accuracy (lower) across a representative analysis during CCS library building. The green line indicates the maximum deviation allowed (± 5ppm for mass accuracy and ± 2% deviation in CCS), and the blue line indicates the area where most of the experimental values were measured (± 2 ppm for mass accuracy and ± 1% deviation in CCS).

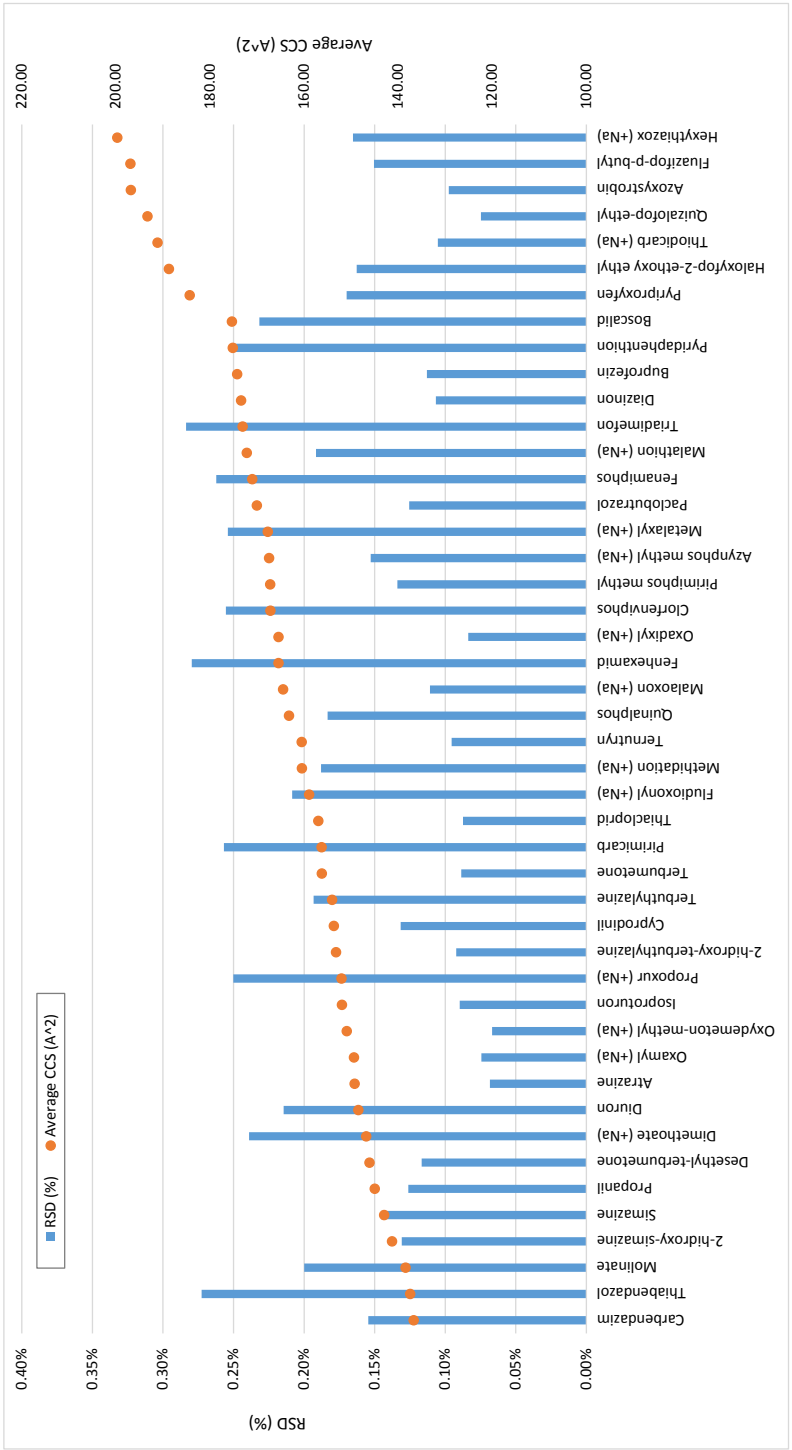

Figure S2. Average CCS value (n=9) and Relative Standard Deviation(RSD) for 46 randomly selected pesticides as an example of the general trend observed in the ion mobility measurement during CCS library building process.

Section S1. Main trends and particularities observed during CCS library development.

Figure S3 shows the CCS value of a certain adduct against the neutral mass (in Da) of the molecule. Even though the CCS value actually corresponds to an ion, and therefore to an *m/z* ratio, the plot is against the neutral mass of the molecule in order to be able to compare CCS values between different adducts of the same molecule. An R-squared correlation coefficient of 0.85 to 0.88 was observed for the correlation of CCS and neutral mass for (de)protonated and sodium adducts, respectively. This highlights the significant dependence of CCS on the mass of the molecule. However, the non-perfect fit to a linear regression highlights that additional molecular parameters affect the spatial conformation of the ion, and in consequence its CCS value. Hence, different CCS values can be observed for similar masses. As an example, in this work a CCS range of 40 $Å^2$ was observed at a neutral mass of approximately 300 Da (*e.g.* over 26 entries comprising 19 proton and 7 sodium adducts between 298-302 Da), in agreement with previous work (1).

Despite the majority of molecules studied followed this trend (CCS *vs* neutral mass), a group of 4 ions in positive mode and 3 ions in negative mode close to mass 800 Da showed a different behaviour, yielding CCS significantly lower than expected (**Figure S3**). The X-ray agents *ioversol*, *iopromide*, *iomeprol* and *iopamidol* have the common chemical backbone of a benzene ring substituted with 3 iodine atoms in positions 1, 3 and 5 and 3 substituents in the remaining positions (**Figure S4**). Instead of resulting in an anticipated higher CCS value because of the larger mass of 3 iodine atoms, this characteristic backbone resulted in a diminished CCS value fostered by the higher atomic density of iodine together with the "wrapping-effect" of the big substituents.

Figure S3 seems to suggest a relationship between the linear regression curves of the protonated, sodiated and deprotonated adducts, *i.e.*, parallel slopes and different intercepts. The CCS of sodiated adducts, for example, may suggest a consistent difference (+6 $Å^2$) compared to the CCS of their corresponding protonated molecules. However, upon closer inspection, (**Figure S6**) this

hypothesis can be rejected. **Figure S6** shows the difference in CCS values for those molecules for which more than one adduct was observed (a total of 299 pairs). It can be seen there is no constant variation of CCS between protonated and sodiated adducts. The addition of a sodium ion to the molecule can either result in a big increase of the CCS value compared with the protonated adduct, as is the case of *flumetasone* ($CCS_{[M+Na]+}$ is 29.33 $Å^2$ greater than $CCS_{[M+H]+}$), or a decrease, as is the case of *heroin* ($CCS_{[M+Na]+}$ is 5.99 $Å^2$ smaller than $CCS_{[M+H]+}$) (**Figure S6a**). This highlighted that CCS values seem to strongly depend on how a molecule can rearrange its structural conformation based on the charge and size of the ion added. Generally, the same behaviour was observed when comparing protonated and deprotonated (**Figure S6b**), deprotonated and formate (**Figure S6c**), and deprotonated and chlorinated adducts (**Figure S6d**).

References for Section S1:

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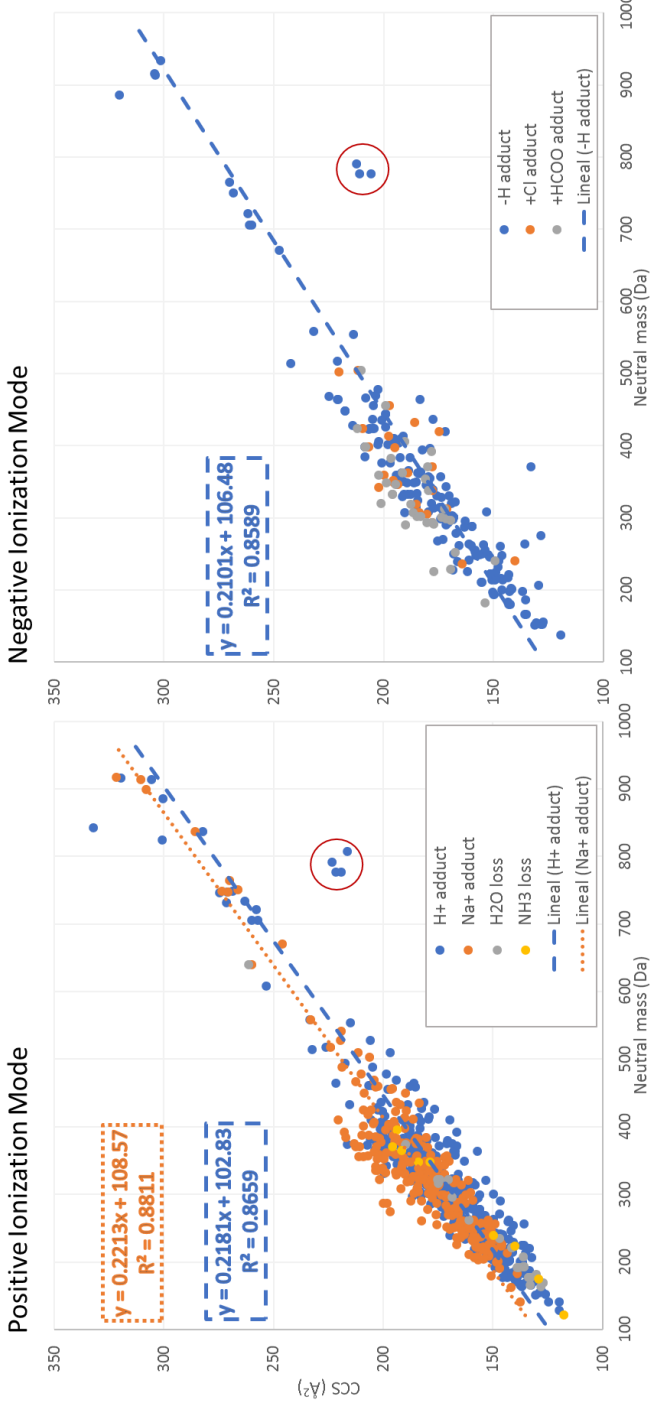

Figure S3. CCS values (Å²) of different adducts versus the neutral mass of the molecule (in Da), for both positive (left) and negative ionization modes (right). The dotted-straight lines represent the correlation between CCS and Neutral mass for the different types for protonated molecules (blue-left line), sodium adducts (orange line) and deprotonated molecules (blue-right line).

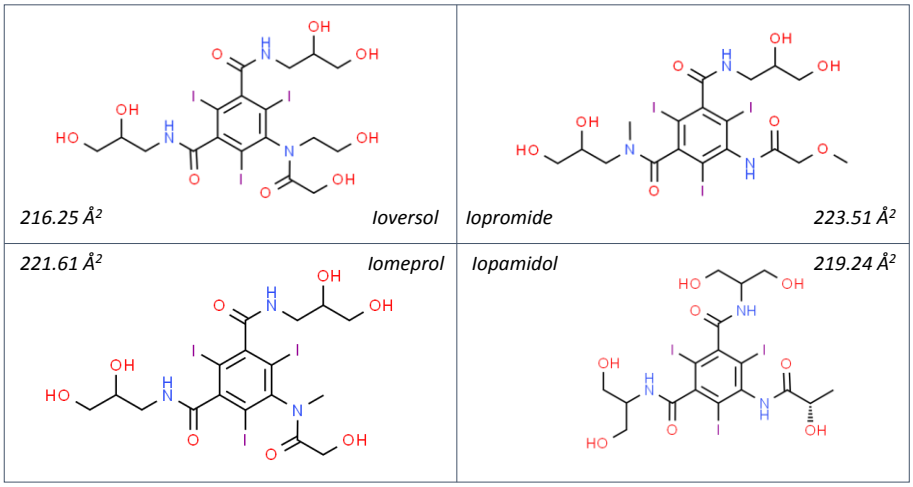

Figure S4. Chemicals structures of X-ray agents ioversol, iopromide, iomeprol and iopamidol.

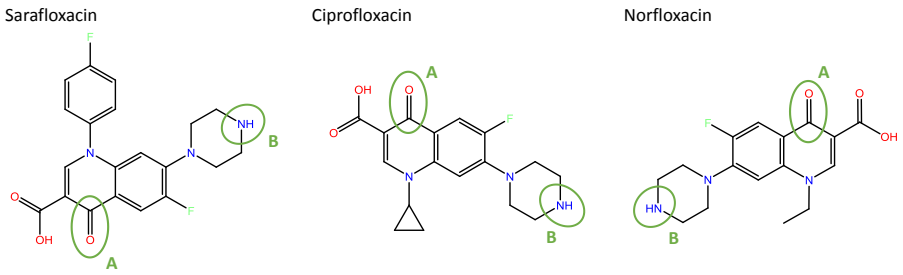

Figure S5. Different protonation sites for sarafloxacin, ciprofloxacin and norfloxacin that result in different protomers separated by IMS-HRMS.

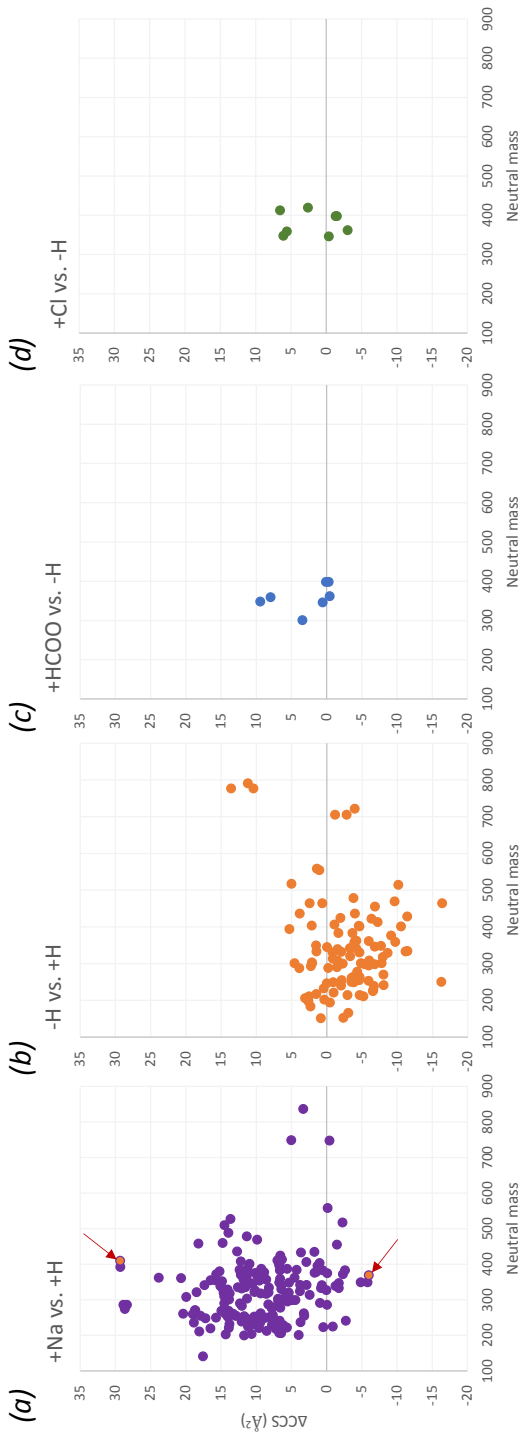

Figure S6. Absolute variation of CCS value observed in molecules showing more than one ionic species. *(a)* Comparison between sodium adduct and protonated molecule, *(b)* Comparison between deprotonated and protonated molecules, *(c)* Comparison between formate adduct and deprotonated molecule, and *(d)* Comparison between chlorine adduct and deprotonated molecule. The variation is plotted against the neutral mass of the molecule (in Da).

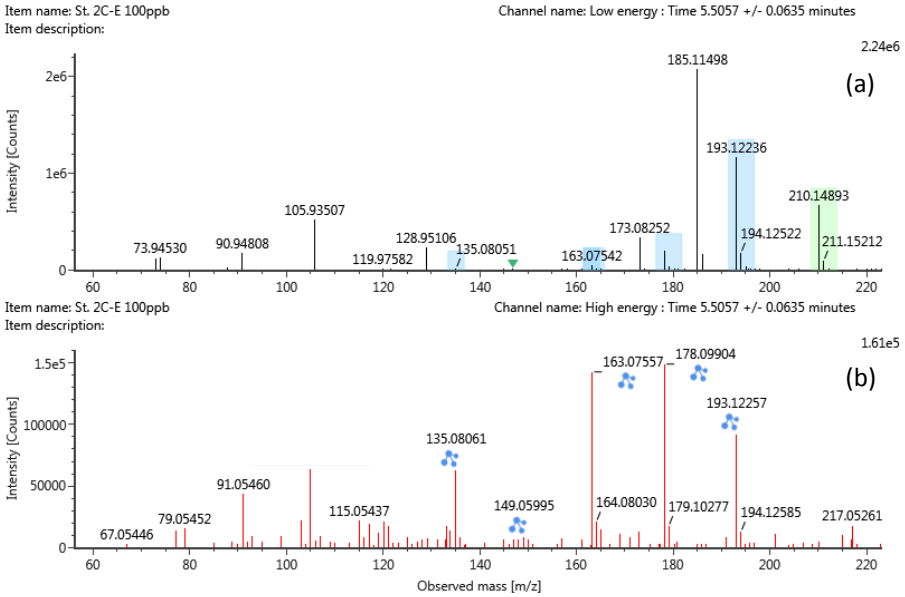

Figure S7. *(a)* LE non-drift time aligned HRMS spectrum for 2C-E and *(b)* HE non-drift time aligned HRMS spectrum for 2C-E .

Chapter 2.2.2. Scientific Article 2

THE RELEVANT ROLE OF ION MOBILITY SEPARATION IN LC-HRMS BASED SCREENING STRATEGIES FOR CONTAMINANTS OF EMERGING CONCERN IN THE AQUATIC ENVIRONMENT

Alberto Celma, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan Vicente Sancho, Karin Wiberg, Lubertus Bijlsma

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The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment

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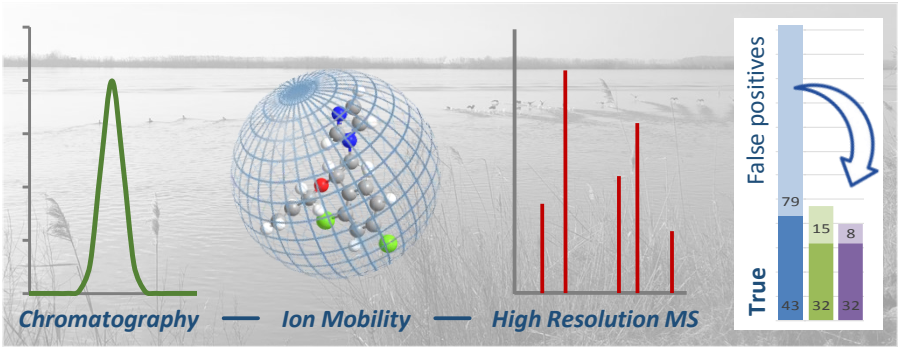
Graphical Abstract



Highlights

- IMS-HRMS facilitates the identification process of organic micropollutants.
- Drift time separation of coeluting ions removes mass spectral interferences.
- CCS value can be used as an additional parameter in the identification criteria.
- IMS reduces the number of false identifications in automated screening workflows.

Keywords

Ion mobility; Screening workflow, Liquid chromatography high-resolution mass spectrometry; Collision cross section (CCS); Environmental analysis.

Abstract

Ion mobility separation (IMS) coupled to high resolution mass spectrometry (IMS-HRMS) is a promising technique for (non-)target/suspect analysis of micropollutants in complex matrices. IMS separates ionized compounds based on their charge, shape and size facilitating the removal of co-eluting isomeric/isobaric species. Additionally, IMS data can be translated into collision cross-section (CCS) values, which can be used to increase the identification reliability. However, IMS-HRMS for the screening of contaminants of emerging concern (CECs) have been scarcely explored. In this study, the role of IMS-HRMS for the identification of CECs in complex matrices is highlighted, with emphasis on when and with which purpose is of use. The utilization of IMS can result in much cleaner mass spectra, which considerably facilitates data interpretation and the obtaining of reliable identifications. Furthermore, the robustness of IMS measurements across matrices permits the use of CCS as an additional relevant parameter during the identification step even when reference standards are not available. Moreover, an effect on the number of true and false identifications could be demonstrated by including IMS restrictions within the identification workflow. Data shown in this work is of special interest for environmental researchers dealing with the detection of CECs with state-of-the-art IMS-HRMS instruments.

1. Introduction

High-resolution mass spectrometry (HRMS) has demonstrated an outstanding potential for target, suspect and non-target screening of contaminants of emerging concern (CECs) in environmental analyses [1–4]. HRMS instruments provide accurate-mass full-spectrum acquisition data that enable to screen for a virtually unlimited number of substances [2,4]. However, mining the large amounts of data generated in MS or MS/MS mode (with information of retention time (RT), mass-to-charge ratio (*m/z*) and peak intensities) is time-consuming, and there is a risk of reporting false positive/negative identifications in complex matrices [5]. Therefore, efforts have been devoted to the development of more sophisticated processing algorithms [6–10], as well as RT or fragmentation predictions tools [11–21], and the incorporation of other techniques to smooth feature identification [22–26].

Ion mobility separation (IMS) coupled to HRMS (IMS-HRMS) is a promising and powerful tool for the (non-)target and suspect analysis of small organic molecules in complex matrices [5,22,27,28]. In brief, IMS separates ionized compounds based on their mobility through a gas (usually N_2 or He) in the presence of an electric field. Such ion mobility mainly depends on the charge, shape and size of the molecule [29,30]. Consequently, IMS theoretically permits the filtering of interfering species such as isomeric or isobaric compounds [5,30–32].

In addition, IMS can provide an extra identification parameter for the confirmation of CECs [5,22]. The drift time (DT), *i.e.* the time it takes for an ionized species to travel through the mobility device, can be converted into a Collision Cross Section (CCS) value based on the measurement of a series of calibrants. CCS values are instrument independent values that are not affected by matrix composition or chromatographic separation [29,30,33]. As a consequence, CCS can be implemented as parameter into the criteria applied for the confirmation of candidate structures [34–36].

Some studies have assessed the precision of empirical CCS measurements in real samples compared to CCS values from reference standards or databases, and

reported that CCS deviations were commonly <2% [29,37–42]. We recently proposed to include CCS into screening criteria for the identification of small molecules in environmental analyses and suggested that the maximum deviation between empirical and expected CCS value should be 2% [34].

One of the most common problems when applying wide-scope suspect and non-target screening strategies for the identification of CECs is the lack of standards for the final confirmation step. Therefore, key information for identification is often absent, such as RT or CCS [2,4]. Although there has been an increase in the number of online available databases of CCS values [34,43–45], the number of entries in those databases is still limited and the available information is biased to common and well-known substances. In this sense, *in-silico* prediction tools of CCS represent a step forward into a more comprehensive incorporation of IMS into the screening workflow [46–49]. Hence, there has been an increase in the number of data-driven machine-learning models with predictive accurateness in the window of ± 3–6% for CCS using Travelling Wave-IMS and Drift Tube-IMS [44,50–55]. Subsequently, several studies have been recently published using prediction of mobility data to gather more confidence in tentative identification when reference standards were unavailable [22,34,50,54].

In this work, an updated workflow with the inclusion of IMS is applied with emphasis on the advantages observed when mobility data is used during data processing and/or for feature identification. Additionally, the benefits of implementing IMS-HRMS for wide-scope screening of CECs in environmental samples are highlighted by means of illustrative examples collected over the experience gathered in different studies considering different scenarios, from target screening (with reference standards), to suspect screening (large list of compounds to be searched) where reference standards are not available.

2. Materials and Methods

2.1. Samples selected as case study

This study shows different examples of the benefits of IMS-HRMS by means of real samples gathered through different research projects. Samples included herein cover the environmental aquatic system *i.e.* influent and effluent wastewater (IWW and EWW, respectively), river water (RW) and lake water (LW) from water bodies in the Mediterranean central littoral of Spain. The extraction methodology followed was adapted from previous studies [56–58]. Briefly, water samples were processed by means of solid-phase extraction (SPE) using generic stationary phases (Oasis HLB, Waters Corporation) with different preconcentration factors (×25 for IWW, ×100 for EWW and ×2500 for RW and LW). All extracts were afterwards reconstituted in 10% MeOH solutions and 1 μL of the final extracts were injected in the UHPLC-IMS-HRMS system.

2.2. Instrumentation

Samples were analysed using a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) connected to a VION IMS-QTOF mass spectrometer, using electrospray ionization (ESI) interface operating in both positive and negative ionization mode. Briefly, chromatographic separation was performed using a CORTECS® C18 2.1 × 100 mm, 2.7 μm fused core column (Waters) at a flow rate of 300 μL min^{-1} over a gradient of 18 min using H_2O (A) and MeOH (B) as mobile phases, both with 0.01% formic acid. MS data were acquired using the VION in $HDMS^e$ mode, over the range *m/z* 50–1000. All data were examined using an in-house built accurate mass screening workflow within the UNIFI platform (version 1.9.4) from Waters Corporation. More details about the instrumentation can be found in **Supporting Information**.

2.3. Target and suspect screening

Targeted screening was performed using an in-house database with 970 entries with information about retention time, mass spectrometric data and CCS values for different adduct species of 556 reference standards in both positive and negative ionization mode. The database is online available for consultation at the

Zenodo repository [59], and contains compounds with different physicochemical properties and uses including pesticides, pharmaceuticals, illicit drugs, hormones, mycotoxins and new psychoactive substances. Detailed information about the development and curation of the database can be found elsewhere [34]. Additionally, an in-house database with 972 substances, for which reference standards were not available at our laboratory, was used for the suspect screening of samples covering parent compounds, metabolites and environmental transformation products of pesticides, pharmaceuticals, hormones and illicit drugs.

The criteria recently proposed for the identification of CECs in environmental analyses by IMS-HRMS [34] was followed in the present work, considering different confidence levels. Briefly, for confirmation purposes at level 1, mass accuracy of both precursor and fragment ions should be < 3 ppm, RT deviation <0.1 min and CCS deviation <2% from the reference standard value. For levels 2 and 3, *i.e.* where no reference standard is available, mass accuracy of precursor and fragments ions should be below 3 ppm from the potential molecular formula. For the prediction of ion mobility data, the model developed by Bijlsma *et al.* [50] with an accuracy threshold for predicted CCS values of 6% was followed.

3. Results and discussion

3.1. IMS-HRMS screening workflow

Screening workflows using HRMS are well established and applied in several studies covering a wide range of aquatic matrices and analytes of interest [2–4,60] with liquid chromatography (LC) being nowadays the separation technique of choice, as most CECs are LC-amenable due to their medium-to-high polarity [2,60]. Nevertheless, large datasets generated in data independent acquisition (DIA) modes make the data processing and final identification of CECs challenging when performing these types of analyses. In this acquisition mode, all ions generated in the ion source are sent to the collision cell for fragmentation without precursor ion selection. This alternation between full-scan and untargeted MS/MS events at different collision energies allows obtaining information on the accurate masses of the (de)protonated molecules as well as their fragment ions. However, DIA data is more complex and, therefore, strategies that curate these convoluted datasets are of high interest to facilitate environmental analysis. Although screening strategies have been improved, IMS was not hitherto included in screening workflows, and the coupling of LC to IMS-HRMS opens new possibilities to monitor CECs in the environment.

Figure 1 outlines a screening workflow from data-acquisition to feature identification in environmental analyses using the 4-dimensional datasets (RT, DT, accurate mass and intensity) generated by LC-IMS-HRMS instruments. In this work we propose to consider the role of IMS at two different stages of the process:

i. **Peak picking, alignment and componentization.** As previously mentioned, IMS theoretically allows the resolution of coeluting substances. Therefore, all ions deconvoluted within a single component of the feature list are required to have the same RT and DT as the precursor ion. These ions include the (de)protonated molecule, commonly predominant in low collision energy (LE) spectra, and ion fragments, commonly observed in the high collision energy (HE) spectra. In this sense, interfering ions coming from other substances

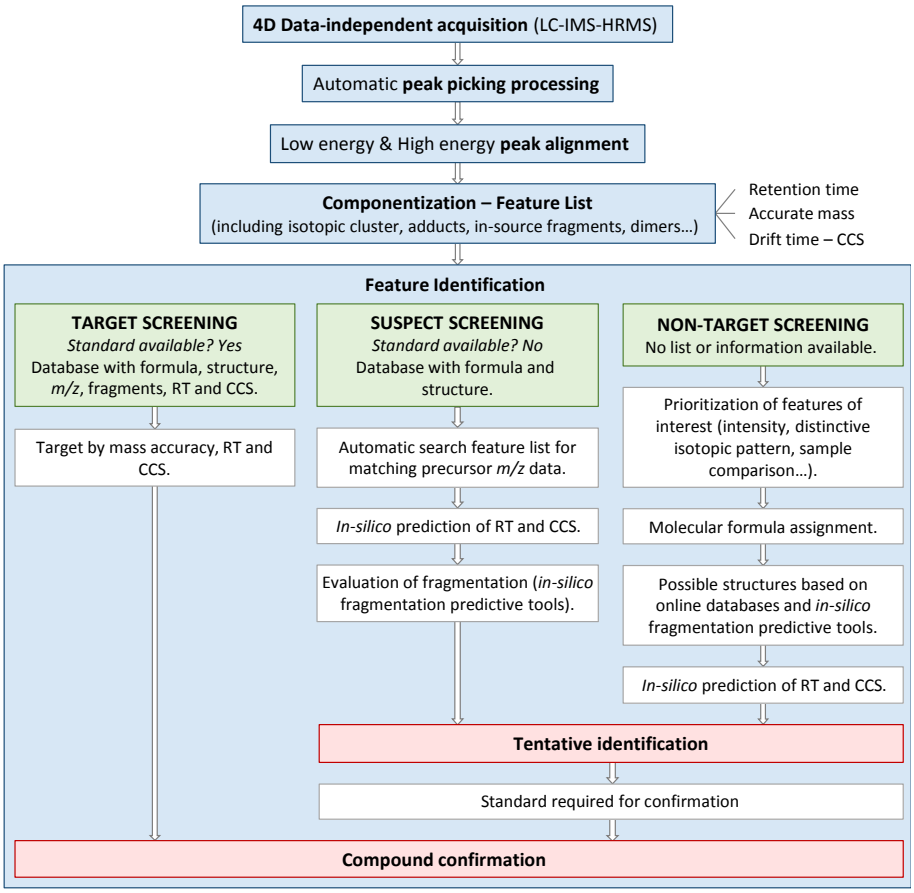


Figure 1. Data-independent acquisition, processing and feature identification workflow for LC-IMS-HRMS target, suspect and non-target screening approaches.

different from the analyte of interest are removed due to their different ion mobility, resulting in much cleaner mass spectra.

ii. **Feature identification.** A remarkable benefit of IMS is the provision of an additional identification parameter. Thus, CCS values can be used for feature identification as an extra point into the confidence gathered for a positive identification. In target screening, the empirical CCS should match with that previously measured from a reference standard with a maximum deviation of 2% [34]. For cases where no reference standard is available, *i.e.* suspect and non-target screening, prediction of CCS is pivotal to benefit from that additional identification point. Although

predicted CCS values are approximations to the real CCS values and cannot be strictly considered (in contrast to real-measured values), the application of CCS prediction can help discarding candidate structures that do not clearly match with the empirical mobility observed. Hence, the identification process of unknown compounds is smoothed and accelerated by the reduction of the number of candidate structures to investigate.

3.2. The application of IMS-HRMS

3.2.1. Drift time alignment – spectral cleaning

During the 4-D peak picking, alignment and componentization process, ions corresponding to the same mass spectrometric feature in both LE (either (de)protonated molecule or adduct ion) and HE (fragment ions) functions should share the same RT and DT as the parent compound. In this way, the automatic processing filters out ionized species that are different from the compound investigated, since all ions exiting the chromatography at a certain time are also separated depending on their mobility. At this point, the location of the mobility device plays an important role for the DT alignment. The mobility device is located after the ionization source and before the collision cell in the majority of IMS-HRMS instruments. Thus, (de)protonated molecules and their corresponding fragment ions have the same DT value in conventional IMS instrument setups. Although less frequent, there are other instrumental configurations where the mobility device is located after the collision cell and in those cases, the benefits of DT alignment may not apply.

Figure 2 is an illustrative example highlighting the spectral cleaning provided by DT alignment of LC-IMS-HRMS data. A positive hit for the pesticide tricyclazole was found at a RT 5.74 min in the 4-D raw data of an analysis of a surface water sample (**Figure 2a**). The conventional HRMS spectra (**Figure 2b**) showed the precursor ion of tricyclazole (*m/z* 194.04310) in the LE spectra (**Figure 2b top**), which is highlighted with a blue arrow. The most abundant peak, *i.e.* base peak with *m/z* 242.28388, corresponds, however, to another

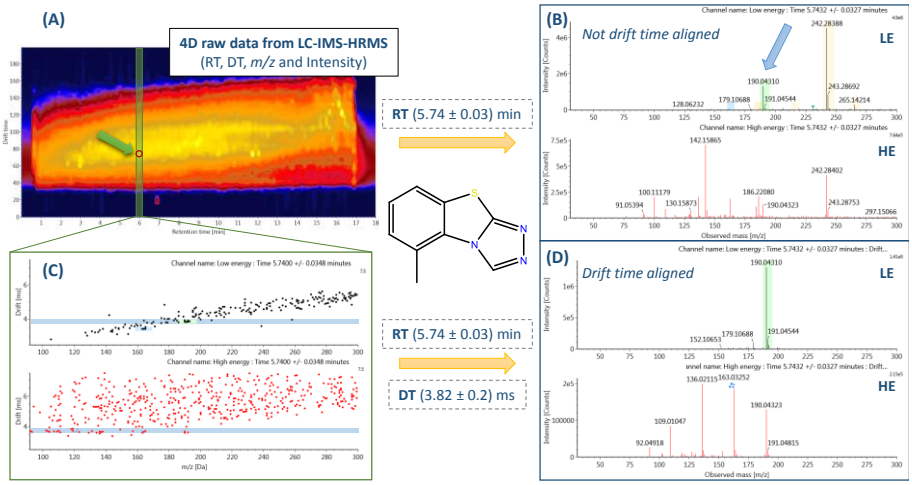


Figure 2. Identification of tricyclazole in surface water. (A) Feature detection of *m/z* 190.04310 at RT 5.74 min and DT 3.82 ms (green arrow); (B) conventional LE (top) and HE (bottom) mass spectra without IMS drift time alignment corresponding to the RT window 5.74 ± 0.03 min; (C) ion mobility separation of co-eluting ions illustrated as red or black dots at the RT window 5.74 ± 0.03 min. Blue highlighted areas are the drift time ranges of 3.82 ± 0.20 ms at LE and HE; (D) LE (top) and HE (bottom) mass spectra with IMS drift time alignment showing only ions within the RT window 5.74 ± 0.03 min and DT window 3.82 ± 0.20 ms.

compound present in the sample. In addition, several ions that were present in the HE spectra (**Figure 2b bottom**), but non-related to tricyclazole hinder the interpretation of the fragmentation pattern of tricyclazole. However, when applying IMS, all ions eluting at 5.74 min could also be separated based on their ion mobility resulting in different values of DT as highlighted by the red or black dots in **Figure 2c**. The DT of tricyclazole (3.82 ± 0.2 ms) and the corresponding fragment ions in this range, represented by the blue highlighted areas, can be aligned.

By means of this drift time alignment, all ions with DT different than those coming from tricyclazole (*i.e.* outside this blue area) are filtered out from the spectra. Thus, in this case, where IMS takes place before the mass fragmentation occurs, all fragments and their associated DT should be the same as the corresponding protonated molecule. Visually, all ionized species out of the blue bands are removed from the spectra, resulting in a drift time aligned MS spectra

(only showing ions within RT 5.74 ± 0.03 min and DT 3.82 ± 0.2 ms) as shown in **Figure 2d**. The drift time aligned MS spectra facilitates interpretation based on the tricyclazole structure, and do match with that observed for the reference standard. Although IMS is known for the extra identification parameter provided by the CCS value [5], the spectral cleaning associated with the drift time alignment is in many cases pivotal for the improvement of screening strategies performance. As shown in this example, the information gathered can be more easily interpreted and, therefore, the data mining of large datasets is notably accelerated, especially in complex-matrix samples.

3.2.2. CCS measurement robustness – additional identification value

Complex matrices can strongly influence the screening outcome by interfering the chromatography and/or the mass spectrometric measurement, even resulting in the reporting of false negative results [2,60,61]. However, as IMS occurs in the gas phase and ionized species do not interact with other substances rather than the gas in the mobility device, matrix does not affect DT measurement and CCS-values are matrix independent. In this section, we aim to highlight how the implementation of IMS-HRMS helped in the identification of *imazalil* in 6 different surface waters affected by strong alteration of the chromatographic retention along the same sequence of analysis.

Table 1 shows the significant variation in the measured RT, while the mass error for the protonated molecule measurement as well as the deviation of CCS remained almost negligible. In this particular case, the RT deviation ranged from 0.14 min up to 0.30 min, while CCS deviation was consistently <2% from standards. Additionally, repeatability of CCS measurements was evaluated across samples showing RSD values < 0.3%. In cases where the RT notably deviates from the standard, European guidelines recommend to spike the sample with the candidate standard to confirm the identity of the compound [62]. However, the additional confidence obtained by the CCS measurement in a single-injection reduces time and costs of spiking and re-injecting the sample, as two separate evidences already exist (MS and CCS). This compound could be

confirmed at level 1 including a note on that RT is deviated, but avoiding the need of re-injecting the sample or further investigation.

Table 1. Variation between the observed *m/z*, detected retention time (RT) and collision cross-section (CCS) values for *imazalil* in six different surface water samples and the reference standard

Sample	Obs. *m/z*	Mass error (ppm)	RT (min)	RT error (min)	CCS (Å²)	ΔCCS (%)
Standard	297.0556 *	-	7.45	-	166.56	-
Water #1	297.0550	-2.0	7.70	0.25	164.86	-1.02
Water #2	297.0555	-0.2	7.60	0.15	164.88	-1.01
Water #3	297.0562	1.9	7.59	0.14	165.20	-0.82
Water #4	297.0563	2.5	7.63	0.18	165.38	-0.71
Water #5	297.0554	-0.7	7.73	0.28	166.15	-0.25
Water #6	297.0562	1.9	7.75	0.30	165.46	-0.66

* Exact mass calculated from the molecular formula ($C_{14}H_{14}Cl_2N_2O$)

This is of special interest in environmental screening strategies where ion mobility, in the authors' opinion, should be evaluated for its potential inclusion as an additional criterion for reliable identification in forthcoming guidelines applied to different fields of analytical research. The example shown in this section illustrates that deviations in RT observed in complex matrix samples may hamper the identification process in wide-scope screening, while the application of CCS provides the extra value needed for confirmation of the identity.

3.2.3. Resolution of isomeric compounds – do they have different CCS values?

Isomeric compounds share the same molecular formula but differ in the arrangement of the atoms, meaning that the overall chemical structure is different. Therefore, HRMS is not able to differentiate between isomeric substances if they share the same fragmentation patterns. In addition, if the

polarity of the isomers is similar, the chromatographic separation may not be able to distinguish between isomers. Yet, isomers could theoretically show different CCS values.

The target database we have previously developed [59] contains information about several pairs of isomeric substances (even some groups of more than 2 isomeric compounds). Among these pairs of compounds, the most challenging ones are those with close RT values as they may pose an extra hurdle for their identification. An example is the pair consisting of *ethiofencarb sulfoxide* (*m/z* 242.08454 | 3.99 min | 146.54 Å^2) and *methiocarb sulfoxide* (*m/z* 242.08454 | 4.39 min | 156.88 Å^2). These pesticides have close RT values that can be easily affected in complex matrices and therefore complicate their identification in real samples. However, their CCS values are significantly different (*i.e.* Δ6.6%), which enables the application of CCS as a distinction tool. A similar example can be found in the group of steroid metabolites constituted by *testosterone glucuronide* (*m/z* 465.2483 | 8.93 min | 221.48 Å^2) and *epitestosterone glucuronide* (*m/z* 465.2483 | 10.18 min | 204.69 Å^2), which have significantly different CCS values (Δ7.6%). On the contrary, *17-α-boldenone* (*m/z* 287.2006 | 9.54 min | 169.32 Å^2) and *17-β-boldenone* (*m/z* 287.2006 | 9.86 min | 171.76 Å^2) have a slight difference in their chemical structure (the α/β orientation of the substituent in a carbon atom) and, as a consequence, they have very close CCS values (Δ1.4%) that do not permit proper differentiation between isomers. Yet, Tian *et al.* [63] could resolve distinct configurations of chiral amino acids (either *D*- or *L*-) by IMS-HRMS by using their chiral ratio to discriminate sample origin [63]. This study demonstrated that IMS can be also of additional value to separate chiral molecules. In these cases, a specific set up of the mobility cell and the use of a chiral gas (*e.g.* (S)-(+)-2-butanol) is often required [64].

When dealing with illegal compounds identification, the differentiation between isomers is even more critical, as it may represent holding legal responsibilities associated with the presence of such banned substances. For example, *methedrone* (*m/z* 194.1176 | 2.34 min | 145.34 Å^2), *3,4-methylenedioxymethamphetamine* (commonly known as *MDMA* or *ecstasy*) (*m/z* 194.1176 | 2.61 min | 145.77 Å^2) and *3-methoxymethcathinone* (*m/z* 194.1176 |

2.39 min | 146.18 Å^2) have all similar fragmentation patterns and very close RT values, which complicates the identification process. Unfortunately, in this case IMS cannot provide additional insight since the CCS values are rather similar, with differences of maximum 0.6%. Contrarily, IMS can help to differentiate *ketamine* (*m/z* 238.0993 | 3.63 min | 148.84 Å^2) from *4-chloro-α-pyrrolidinopropiophenone* (*m/z* 238.0993 | 4.02 min | 154.04 Å^2) since their CCS value differs with 3.6%.

Separation of protomers by means of IMS-HRMS has been evaluated elsewhere [65] highlighting the possibility of resolving different protomers for quinolone antibiotics. However, little is still known about the resolution needed to separate the protomers formed and the potential impact in screening approaches (signal reduction, false negatives/positives, etc.). This is a challenging issue and further studies need to be conducted towards the evaluation of protomers determination in complex matrices.

As shown, the potential of IMS for the resolution and unambiguous identification of isomeric substances and protomers is promising, but there are still some limitations mainly due to the low resolution power of commercial IMS instruments. Harvesting the extra benefits provided by IMS would require the implementation of higher resolution IMS systems [66], and it is the hope of the authors that forthcoming developments in IMS instruments will accomplish pursue that objective.

3.3. How does IMS affect the number of false positives in automated screening workflows?

A common problematic issue in applying automated screening workflows is the possible reporting of misidentifications. In this section, the potential of IMS-HRMS to minimize the number of false positives in environmental water samples is explored and illustrated with some examples.

The collaborative project entitled "*Effect-directed analysis as a tool towards a non-toxic environment – identification of mixture effects and toxicity drivers in*

water (DANTE)" aims to produce a robust strategy to assess environmental toxicity through the combination of toxicological and chemical analyses. Consequently, the screening strategy to be applied within this project needs to be carefully evaluated to produce reliable and intercomparable results. Four water samples (influent and effluent wastewater, river water and lake water) were spiked for quality control purposes, and are shown as a case study for the evaluation of the amount of false positive identifications using an automated screening workflow by LC-IMS-HRMS. Samples were spiked with a mixture of 59 compounds consisting of pesticides and pharmaceuticals prior to sample treatment (SPE). The whole list of standards that were used for spiking is available in **Table S1** of the **Supporting Information** (SI). After SPE, the samples were screened for these substances applying the following criteria: response >1000 counts; $[M+H]^+$ error <3 ppm; RT error <0.1 min; and CCS deviation <2%. Additionally, to assess the benefits of the prediction of CCS values, a suspect screening was applied for the same compounds but with predicted CCS [50] as if no reference standards and, thus, experimental CCS were available. Briefly, this predictive model makes use of an artificial neural network (ANN) to predict CCS values from the input of 8 molecular descriptors. The threshold for the predicted CCS (CCSPred) data (**Table S1** in **SI**) was established at < 6% (*i.e.* the prediction accuracy at the 95th percentile reported by Bijlsma *et al.*).

Figure 3 shows the true identifications (solid color) and false positives (pale color) when applying different criteria. True identifications were confirmed based on MS, RT and CCS; the same criteria was used to discard false positives. In general, the application of mass accuracy restrictions only (blue colored bar) was the criterion that rendered the maximum number of true identifications. However, the number of false positives (pale blue) and the time needed for data revision were also high. The inclusion of CCS into the criteria for identification (MS and CCS, red colored bar) did not result in a reduction in the number of true identifications (solid red), but it did reduce the number of false positives facilitating data revision.

The case is different when the conventional criteria in LC-HRMS for identification (MS and RT, green colored bar) is applied. In this case, a decrease

in the number of true identifications was observed, mainly due to RT deviations. The inclusion of this parameter in the automated screening criterion strongly affected the identification performance. Yet, the number of false potential positives was also reduced by RT restrictions. As can be seen from **Figure 3** (blue, red and green bars), the reduction in false positive identifications when applying a RT filter is much higher than when considering CCS values. The latter could be somewhat correlated to the molecular mass, although CCS values for the same *m/z* sometimes ranged of more than 35 Å^2 [50]. When applying LC-IMS-HRMS criteria for the identification (MS, RT and CCS, purple colored bars), the effect of RT on the number of true/false positives is also limiting the performance of the screening in this case and, therefore, hindering the benefits of CCS. However, the inclusion of CCS permitted the removal of some false positives (pale purple) in comparison to only use MS and RT criteria (pale green). At this point, it is worth mentioning that less restrictive thresholds for RT compliance could be considered when analyzing complex matrices in order to

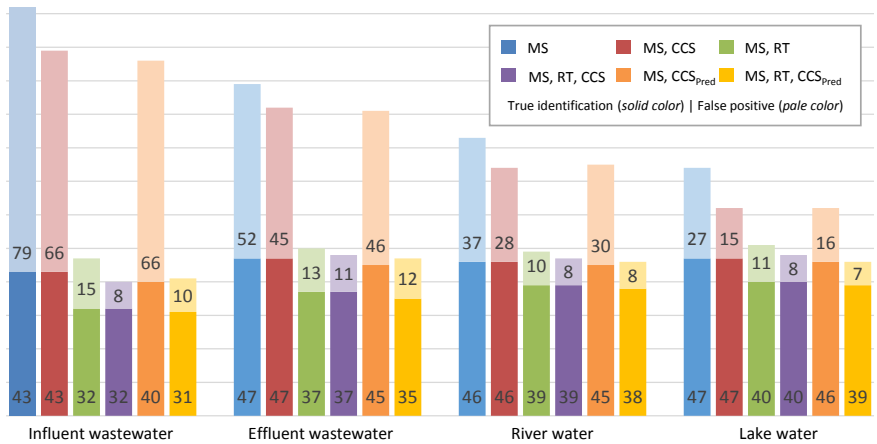


Figure 3. Effect of identification criteria applied on the number of true and false identifications over a set of 59 standards spiked in real samples (*i.e.* influent and effluent wastewater, river and lake surface water). True identifications in solid color and false identifications in pale color. MS: accurate mass deviation < 3 ppm, RT: empirical retention time < 0.3 min from standard, CCS: empirical CCS < 2% deviated from standard, CCS_{Pred}: empirical CCS value < 6% deviated from prediction.

avoid reporting false negative identifications. In this scenario of wider limits for RT, CCS can give an additional and complementary confidence in the identification.

When a suspect screening is applied using only predicted CCS values and no empirical data, the number of true identifications (MS, CCS_{Pred}, orange colored bar) was, in some cases, slightly reduced in comparison to experimental CCS values from standards (solid red). Again, when including RT with CCS_{Pred} as criteria, the effect of RT filtering dominates the screening performance and reduces the number of potential positives to be investigated. Despite the effect of RT filtering, predictive tools for ion mobility data are indeed helpful when no reference standard is available. Thus, predicted CCS values can be used as an additional value for the screening of CECs in complex matrices.

Most remarkably, the inclusion of CCS into the identification criteria for both target and suspect screening strategies was not detrimental for the screening performance, contributing to reduce the number of false positives without affecting the number of false negatives. Contrarily, RT notably affected the performance of the screening as the chromatography is much more affected by complex matrices components. These examples highlight the fact that automated screening workflows should be carefully applied and require the critical assessment of an experienced analyst in order to differentiate true identifications from false positives and to report curated and high quality results.

4. Conclusions

LC-IMS-HRMS is still scarcely used in the analysis of CECs in environmental samples, and therefore little is known about the benefits and drawbacks of the application of this technique in this specific field. In this study, an overview of the potential of LC-IMS-HRMS, with a discussion on the main pros and cons, is presented, making use of selected examples to illustrate its application to the screening of CECs in a wide range of water samples. The mass spectra cleaning provided by DT alignment, the value of CCS as additional identification parameter as well as the potential separation of isomeric and isobaric substances are some of the main benefits one can harvest from IMS-HRMS. Additionally, CCS prediction is a powerful strategy to improve the suspect and non-target screening approaches by reducing the number of candidates to investigate as well as providing extra evidence on tentative identifications. The effect of including CCS restrictions within the criteria for compound identification has been also assessed yielding a better performance than RT in large screenings. As shown, empirical CCS and predicted CCS values did not reduce the number of true identifications but the number of false positives to be investigated. Consequently, the data revision process is notably facilitated by eliminating candidates that do not match with the expected data, reducing the time consumed and increasing the throughput of the strategy.

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Supporting information

This section contains detailed information on the instrumentation used for the study as well as predicted CCS data for the set 59 compounds spiked to the samples.

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Supplementary data

Section S1

A Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) connected to a VION IMS-QTOF mass spectrometer, using electrospray ionisation (ESI) interface operating in both positive and negative ionisation mode was used for the analysis of samples. Chromatographic separation was performed using a CORTECS® C18 2.1 x 100 mm, 2.7 µm fused core column (Waters) at a flow rate of 300 µL min^{-1}. Gradient elution was performed using H_2O (A) and MeOH (B) as mobile phases, both with 0.01% formic acid. The initial percentage of B was 10%, which was immediately linearly increased to 90% over 14 min, followed by a 2 min isocratic period, and then returned to initial conditions (at 16.1 min) with a 2 min equilibration of the column. The total run time was 18 min. The injection volume ranged from 1 to 5 µL.

A capillary voltage of 0.8 kV for positive and 2.5 kV for negative ionization mode and a cone voltage of 40 V were used. The desolvation temperature was set to 550 °C, and the source temperature to 120 °C. Nitrogen was used as the drying gas and nebulizing gas. The cone gas flow was 250 L h^{-1} and desolvation gas flow of 1000 L h^{-1}. The column temperature was set to 40 °C and the sample temperature to 10 °C. MS data were acquired using the VION in HDMSe mode, over the range *m/z* 50-1000, with N_2 as the drift gas, an IMS wave velocity of 250 m s^{-1} and wave height ramp of 20-50 V. Leucine enkephalin (*m/z* 556.27658 and *m/z* 554.26202) was used for mass correction in positive and negative ionization modes, respectively. Two independent scans with different collision energies were acquired during the run: a collision energy of 6 eV for low energy (LE) and a ramp of 28-56 eV for high energy (HE). A scan time of 0.3 s was set in both LE and HE functions. Nitrogen (≥ 99.999%) was used as collision-induced dissociation (CID) gas. All data were examined using an in-house built accurate mass screening workflow within the UNIFI platform (version 1.9.4) from Waters Corporation.

Table S1. List of compounds spiked in water samples for the assessment of true/false identifications. Empirical CCS values for [M+H]+ were obtained from standards and predicted CCS values were calculated using the predictive model developed by Bijlsma *et al.* Deviation was calculated between the empirical and the predicted CCS values.

Item Name	CCS Emp. (Å²)	CCS Pred. (Å²)	CCS dev (%)
2-hydroxy-terbuthylazine	153.11	148.80	-2.8%
4-Hydroxy omeprazole sulfide	174.93	170.97	-2.3%
Acetamiprid	152.21	144.71	-4.9%
Alprazolam	171.94	167.00	-2.9%
Atorvastatin	233.34	234.02	0.3%
Atrazine	149.26	144.41	-3.2%
Azithromycin	268.72	296.38	10.3%
Carbamazepine	149.11	150.89	1.2%
Carbaryl (Na adduct)	147.98	141.02	-4.7%
Chlorpyrifos (ethyl)	163.12	159.94	-1.9%
Ciprofloxacin (protomer I)	175.38	177.02	0.9%
Ciprofloxacin (protomer II)	188.89	177.02	-6.3%
Clarithromycin	271.25	273.66	0.9%
Clindamycin	202.49	201.92	-0.3%
Clothianidin	151.65	143.88	-5.1%
Deethylatrazine	139.64	134.07	-4.0%
Deethylterbumeton	146.07	143.32	-1.9%
Deisopropylatrazine	132.85	129.94	-2.2%
Desethyl terbuthylazine	144.71	138.21	-4.5%
Diclofenac	156.92	156.97	0.0%
Diuron	148.38	141.54	-4.6%
Enalapril	187.96	198.71	5.7%
Flumequine	150.58	153.44	1.9%
Furaltadone	173.06	173.84	0.4%
Gabapentin	139.70	134.98	-3.4%
Imazalil	166.56	166.24	-0.2%
Imidacloprid	153.91	150.24	-2.4%
Iopromide	223.51	210.15	-6.0%
Irbesartan	202.81	208.29	2.7%
Lincomycin	201.18	199.22	-1.0%

Item Name	CCS Emp. (Å²)	CCS Pred. (Å²)	CCS dev (%)
Linuron	151.01	145.45	-3.7%
Lorazepam	166.11	162.29	-2.3%
Losartan	200.49	201.85	0.7%
Metalaxyl	160.08	168.71	5.4%
Methiocarb sulfoxide	156.88	150.93	-3.8%
Metolachlor	159.39	166.56	4.5%
Metoprolol	172.54	170.78	-1.0%
Metronidazole	131.04	132.08	0.8%
Norfloxacin (protomer I)	171.88	174.12	1.3%
Norfloxacin (protomer II)	187.60	174.12	-7.2%
Pantoprazole	184.38	182.27	-1.1%
Paracetamol	130.56	128.67	-1.4%
Phenazone	135.59	138.04	1.8%
Primidone	147.25	146.56	-0.5%
Propamocarb	144.69	144.22	-0.3%
Pyridaphenthion	175.12	172.99	-1.2%
Roxithromycin	282.33	294.59	4.3%
Salbutamol	159.93	159.33	-0.4%
Simazine	143.00	139.26	-2.6%
Sulfadiazine	151.96	150.04	-1.3%
Sulfamethoxazole	152.61	150.42	-1.4%
Tebuconazole	166.80	173.06	3.8%
Terbumeton	156.21	155.14	-0.7%
Terbuthylazine	153.99	149.53	-2.9%
Terbutryn	160.48	156.97	-2.2%
Thiabendazole	137.44	133.11	-3.1%
Thiacloprid	156.97	146.74	-6.5%
Thiamethoxam	158.16	154.08	-2.6%
Tramadol	161.30	166.12	3.0%
Trimethoprim	172.89	170.14	-1.6%
Venlafaxine	171.86	171.31	-0.3%

2.3. Discussion

The hyphenation of IMS to HRMS has been explored in the field of biomolecules (Chouinard et al., 2016; Hofmann et al., 2015; Hofmann and Pagel, 2017; Jeanne Dit Fouque and Fernandez-Lima, 2019; Rue et al., 2020), metabolomics (Luo et al., 2020; Paglia et al., 2021), lipidomics (Paglia et al., 2021), food analysis (Goscinny et al., 2019; Hernández-Mesa et al., 2019, 2017; Merel et al., 2019), exposome (Metz et al., 2017), and forensics (King et al., 2019; Lian et al., 2018; Mardal et al., 2018; Radchenko et al., 2020; Rister and Dodds, 2020b; Ross and Xu, 2021). However, there is a need to explore the applicability of this technique to the analysis of OMPs in environmental samples as well as to demonstrate the benefits of such application. In this sense, different key points have been addressed in **scientific article 1** and **2** that are going to be jointly discussed in the following paragraphs.

Collision cross section measurement as an additional identification parameter for environmental analysis

The establishment of well-defined criteria accepted by the scientific community to report the confidence on the identification of candidates in screening

strategies is essential for a harmonized dissemination of results. Schymanski *et al.* proposed a 5-level criteria for the identification of small molecules using chromatographic separation coupled to HRMS going from level 5 – *exact mass of interest* (lowest confidence) to level 1 – *confirmed structure* (highest confidence) (Schymanski et al., 2014a). This classification was rapidly accepted within the environmental scientific community and became the golden standard for reporting screening results for both targeted, suspect and non-target screening methodologies (Alygizakis et al., 2018; Bletsou et al., 2015; Chiaia-Hernandez et al., 2017; Menger et al., 2020; Schymanski et al., 2015). However, as previously explained, this classification relies on the evidence gathered with chromatography and HRMS with no mention of IMS. In **scientific article 1**, the incorporation of IMS together with HRMS and chromatographic separation for the identification of candidates was proposed following the scheme of the criteria previously presented by Schymanski *et al.* (Schymanski et al., 2014a). Additionally, level 1 – *confirmed structure*, which represents the ideal situation with chromatographic, IMS and HRMS evidence being confirmed with a reference standard, was complemented by a scoring system (**Table 2.1**). The finding of the (de)protonated molecule (or corresponding adduct) with an appropriate mass accuracy counts 1 identification point (IP), and 2.5 IP can be summed if two or more HRMS fragments are observed in accordance with the fragmentation spectra of the reference standard. However, the weight of RT (1 IP) and CCS (1.5 IP) in the scoring system is not balanced. This is explained by the fact that RT can easily be affected by matrix endogenous components resulting in deviations from reference standard higher than 0.1 min. Yet, the chance of having deviations greater than 2% in CCS measurement is low because of the robustness of the measurement *i.e.* matrix does not affect the DT. Therefore, RT and CCS should not be weighted uniformly since a deviation in RT is more likely to happen than in CCS. Thus, a deviated RT alone is not enough to question the candidate identity especially when the empirical CCS is matching the corresponding standard.

Table 2.1. Scoring system for Level 1 candidate confirmation in targeted analysis.

Evidence line	**Identification points**
Empirical MS information matches the reference standard	1 IP
Empirical RT information matches the reference standard	1 IP
Empirical CCS information matches the reference standard	1.5 IP
Two or more matching HRMS fragments	2.5 IP
Minimum IP for Level 1* identification with CCS	5 IP
Ideal IP for Level 1 identification	6 IP

A minimum of 5 IP is proposed to confirm a candidate identity, which would correspond to 2.5 IP from fragment ions, 1.5 IP from CCS accuracy and 1 IP from RT or MS accuracy of (de)protonated molecule. However, even Level 1 identifications can be sometimes irresolute and challenging, when RT or MS exceed the deviations, namely mass accuracy can be strongly affected by ion intensity and RT by matrix-endogenous compounds. In these cases, and when deviations are slightly over the established thresholds, the identity can be considered as confirmed at Level 1*, followed by a brief explanation on the cause of the deviation.

As shown in **Table 1** of **scientific article 2**, deviations in RT over a sequence of chromatographic runs can be observed because of matrix endogenous compounds. In this case, imazalil was detected in 6 different samples with the RT being clearly deviated from the reference standard. However, the CCS values observed for imazalil in the 6 samples were deviated from reference standard less than 1%, clearly below the threshold of 2% established for CCS accuracy. The robustness in CCS measurement increased the confidence on the identification of imazalil in those surface water samples at Level 1*. However, if conventional HRMS instruments (with no IMS) would have been used, imazalil could only resulted in a tentative identification and further efforts would have been necessary to confirm their identity. With the inclusion of IMS into the criteria, imazalil could be confirmed without the need of reinjecting the sample

and, therefore, reducing time and costs and increasing the throughput of samples. This new incorporation of multiple lines of evidence into identification confidence schemes arises several benefits for compound identification.

Development and availability of CCS databases on OMPs

The application of IMS-HRMS to study OMPs in environmental samples is still limited. Consequently, the availability of databases containing information of empirical CCS values for environmentally relevant chemicals is also reduced. However, some compendium of CCS values have been published and open the future for further data sharing among the scientific community (May et al., 2017; Picache et al., 2019; Ross et al., 2020; Zhou et al., 2020). At the moment, the vast majority of CCS databases comprise both empirical and modelled CCS values without including other relevant information *e.g.* MS fragmentation. **Scientific article 1** presented the first database publicly available containing information about *m/z* values for both (de)protonated molecule and fragment ions, RT and CCS. This database presented information of about 1,000 chemical species and it is also available on the Zenodo online and open access repository (Celma et al., 2019). To facilitate the implementation and use of the data provided into third-party laboratories, the database was imbedded in PubChem as individual annotations for the measured reference standards entries. Recently, CCS values gathered in CCSbase (www.ccsbase.net) by Ross *et al.* have been also incorporated into PubChem (Ross et al., 2020) remarking the high interest of the scientific community to release and share relevant, helpful and impactful data for other studies.

Although there is high interest in developing online databases for CCS values, there is an increasing concern on the comparability of CCS values measured using different IMS instruments or techniques. Stow *et al.* shed light on the interlaboratory comparability of CCS values measured using the same technique. In this case, authors evaluated the CCS values measured using DTIMS instruments, finding average deviations of 0.29 % for a wide range of chemical families, masses and charges in both positive and negative ionization modes

(Stow et al., 2017). It is critical for those comparisons that the same drift gas is used in all instruments. A similar study was conducted by Righetti *et al.* for the evaluation of differences in CCS values for a set of mycotoxins over three different TWIMS instruments using same calibrant solutions (Righetti et al., 2020). Authors did not find significant differences in the CCS values measured and concluded that databases developed using TWIMS can be used across different TWIMS instruments when same calibrants are used. In another study, Hinnenkamp *et al.* compared CCS values determined by TWIMS and DTIMS (Hinnenkamp et al., 2018). Provided that both instruments were operating using the same drift gas (*i.e.* N_2) and calibrants, authors found good correlations between CCS values with no relevant offset between the two techniques. The average deviations between instruments were 1.0 % difference for protonated molecules and 1.1 % for sodium adducts. Yet, 7 % of the protonated molecules and 13 % of sodium adducts showed difference above 2 % between the instruments (Hinnenkamp et al., 2018). Based on these results, CCS values measured with different instruments and/or techniques can be used for comparison, but cautions should be taken to gather additional information about the instrument and method followed for CCS measurement. It is, therefore, pivotal that CCS databases also present the instrumental details used for data acquisition.

Signal filtering by IMS-HRMS

The intrinsic separation of coeluting ions provided by IMS brings an extra advantage of IMS-HRMS as the complexity of mass spectra acquired (especially in DIA mode) can be often reduced by the separation of background ions that do not correlate to the ion of interest (Dodds and Baker, 2019). This separation is of great help for the increase of signal-to-noise ratio in the particular case when background ions are of high intensity. IMS can be also used to remove contaminating signals in order to obtain higher quality mass spectra as highlighted in **Figure 6** of **scientific article 1** and **Figure 3** of **scientific article 2**. The quality of the spectra, especially for high energy collision spectrum, is

comparable to MS/MS acquisitions. Such improvement in quality provided by IMS-HRMS is of great benefit to facilitate data evaluation specially in suspect and non-target screening of highly complex matrices such as surface water and wastewater.

The drift time alignment, that provides clean mass spectra, is often automatically performed. In the case of the TWIMS instrument used in this thesis, the manufacturer software for data acquisition, processing and revision performs a peak deconvolution in which the DT of the analyte of interest is measured. Then, ions in the LE and HE spectra sharing the same DT value are aligned while the remaining ions are eliminated. **Figure 2.1** depicts the magnitude of the signal filtering for a targeted detection of thiabendazole in a river water sample by UHPLC-IMS-HRMS. Co-eluting compounds are IMS separated resulting in the scattering of ions shown in **Fig. 2.1 (A1)** for LE and **Fig. 2.1 (A2)** for HE functions. During the data processing, the software automatically extracts ions with approximately the same DT as the $[M+H]^+$ for thiabendazole (shown as black dots) and discard the remaining ions with different mobilities (shown as grey dots). This ion selection results in the DT aligned mass spectra shown in **Fig. 2.1 (B1)** for LE and **Fig. 2.1 (B2)** for HE. Again, the black MS signals correspond to the ions selected during DT alignment, while the grey ones are the ions with different mobilities. As shown by this example, the DT-aligned MS spectra is much cleaner than the one provided by non-DT aligned data. This signal filtering is particularly advantageous for the analysis of small molecules in complex matrices due to high quality of the spectra obtained comparable to reference standard injections.

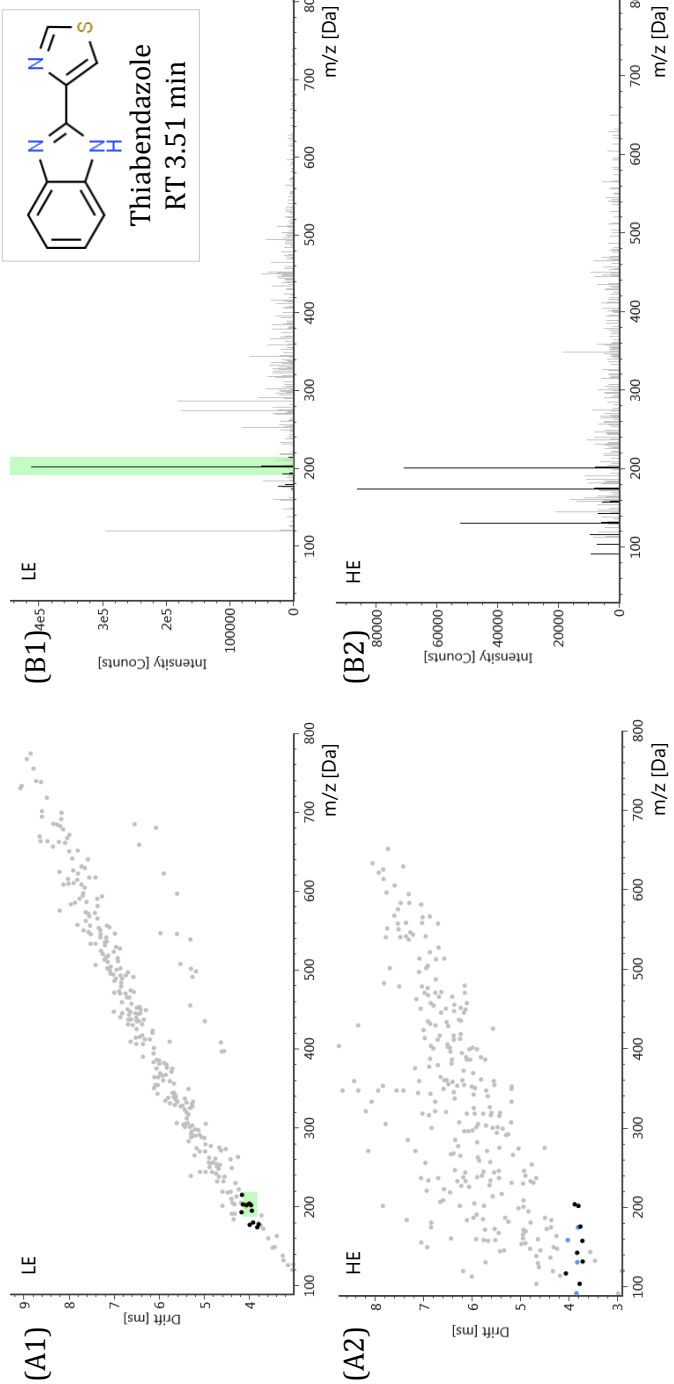


Figure 2.1. Mass spectral cleaning provided by IMS-HRMS drift time alignment exemplified by thiabendazole detection in surface water sample. Grey colored ions correspond to the not drift time aligned data and black colored signals correspond to the drift time aligned data . (A) Detected ions (x-axis) separated based on their mobility – drift time (y-axis) for low energy (LE) spectrum (A1) and high energy (HE) spectrum (A2). (B) Mass spectra for LE (B1) and HE (B2) functions.

However, losses of signals of interest can also occur due to DT alignment. In the event that the analyte of interest suffers from in-source fragmentation, the compound arrives at the mobility device split into more than one ion. Thus, they may have different mobilities (*i.e.* DT) and, therefore, the automatic DT alignment would not assign the fragments originated from 'in-source' species to the (de)protonated molecule of the candidate of interest. This can result in missing diagnostic fragments in the high collision energy spectrum and, therefore, translate into problematic identifications. A potential solution found during this thesis consists on the inclusion of known in-source fragments of target analytes as a secondary adduct specimen in the corresponding entry of the database. Although they are not real adducts, the software understands the species as a different adduct apart from the (de)protonated molecule and, consequently, DT alignment considers fragment ions coming from both species.

Isomeric compound resolution

The distinction of isomeric compounds can be challenging because fragmentation spectra are often very similar and may fail to provide diagnostic evidence for unequivocal identification. The separation of coeluting ions based on shape, size and charge offered by IMS opens the possibility for the resolution of such species by providing a complementary separation (Dodds and Baker, 2019; Kaufmann et al., 2020).

Several studies have evaluated the resolution of isomeric substances by means of IMS-HRMS demonstrating an outstanding potential for such application mainly for biomolecule analysis (D'Atri et al., 2018; Delvaux et al., 2020; Dodds and Baker, 2019; Rister and Dodds, 2020a; Wu et al., 2020), polymers (Austin et al., 2021; Liénard et al., 2020) and small molecules (Kaufmann et al., 2020; Knudsen et al., 2021; May et al., 2020). However, as demonstrated in **scientific article 2**, the resolution of isomeric substances for small molecules is more challenging due to the small differences in the CCS values measured (Kaufmann et al., 2020). While some substances may have different CCS values that permit their unequivocal identification, they also show different RT and MS

fragmentation. Thus, IMS acts as an additional parameter for identification rather than physically separating purely coeluting isomeric substances.

During CCS library development in **scientific article 1**, different protomers for quinolone antibiotics could be observed. The different protonation sites occurring in norfloxacin, ciprofloxacin and sarafloxacin mimics the situation of isomeric substances having equal RT and similar fragmentation spectrum. **Figure 2.2** shows the mobilogram for norfloxacin protomers with two different drift peaks resolved below 50 % from baseline (indicated as a red line). The mobility separation observed can be considered as an approximate threshold upon which resolution of isomeric substances is accomplished. Hence, the minimum required difference in CCS values for two mobility peaks to be resolved at half maximum height is approximately 8 %. Although separation by ion mobility cannot be accomplished for many isomeric compounds due to the relative low resolving power of these first generation IMS instruments, it has been a starting point for new technical developments and improvements, such as the recently developed cyclic-IMS instruments. In this IMS instrument the length of the mobility device can be optimized for particular compounds, which will increase ion mobility resolving power enabling a more comprehensive and accurate isomeric substances resolution (D'Atri et al., 2018; Dodds and Baker, 2019; Kaufmann et al., 2020; McCullagh et al., 2019).

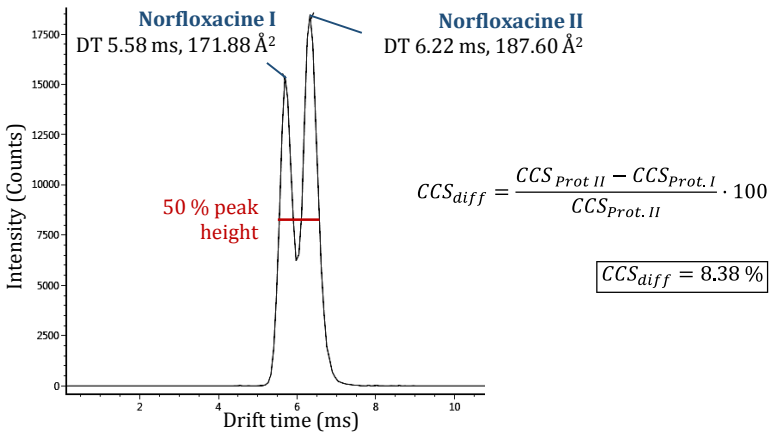


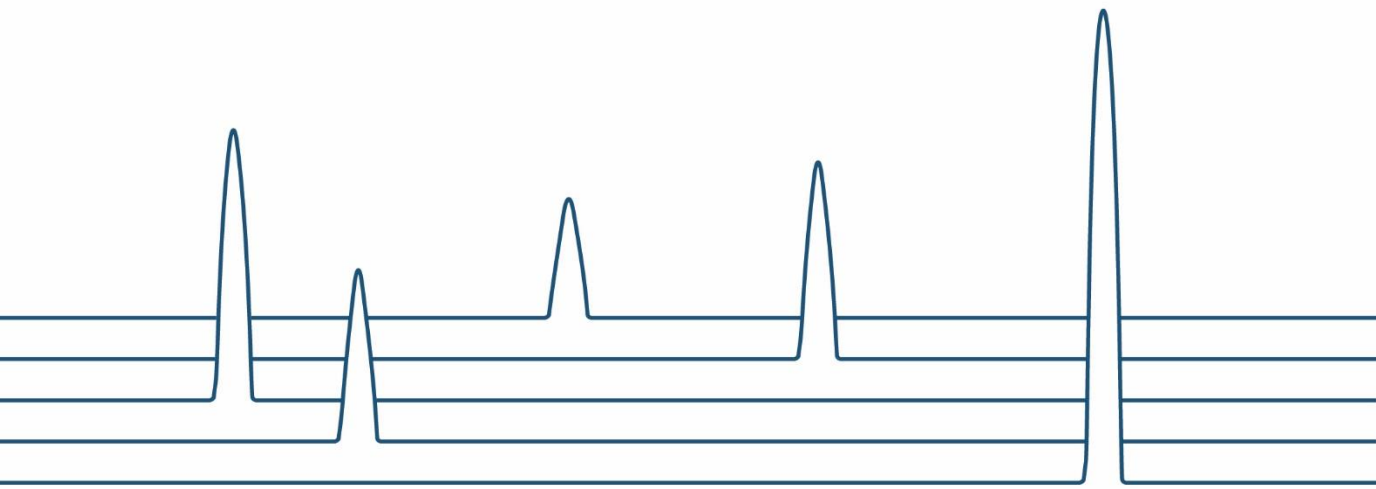
Figure 2.2. Norfloxacin protomers separated by IMS (mobilogram). Calculation of CCS difference for resolution of purely coeluting ions at a minimum of 50 % of peak height.

False positive rate reduction by IMS-HRMS

As previously mentioned, the low concentration of CECs and the high complexity of environmental matrices often overcomplicate data interpretation. Also, the application of automated screening workflows (*i.e.* peak deconvolution, DT alignment and feature identification) may increase the likelihood of misidentifications because of matrix interferences. Nevertheless, the advantages of IMS-HRMS as explained earlier, facilitate data revision by minimizing the number of false positive findings in environmental water samples. As demonstrated in **scientific article 2** with the targeted analysis of a set of spiked substances in a range of environmental matrices (influent and effluent wastewater, river water and lake water), the inclusion of CCS into the identification criteria do not affect the number of true identifications observed. Additionally, it also contributes to reduce the number of false positives by 15–46 % (depending on the matrix, more details in **Figure 3** of **scientific article 2**). RT, however, notably affects the performance of the screening as the chromatography is much more affected by complex matrices components. Therefore, establishing strict thresholds for automated screening workflows can be problematic and needs to be carefully thought-out. Finally, the critical assessment of an experienced analyst is always necessary in order to differentiate true identifications from false positives and to report curated and high quality results.

CHAPTER 3.

INTERPOLATIVE AND PREDICTIVE TOOLS FOR THE REFINEMENT OF SUSPECT AND NON-TARGET SCREENING STRATEGIES



Chapter 3. Interpolative and predictive tools for the refinement of suspect and non-target screening strategies

3.1. Introduction

3.2. Scientific Articles

3.2.1. Scientific article 3.

"Development of a Retention Time Interpolation scale (RTi) for liquid chromatography coupled to mass spectrometry in both positive and negative ionization modes"

Journal of Chromatography A 1568 (2018) 101-107

3.2.2. Scientific article 4.

"Prediction of Collision Cross-Section values for small molecules: Application to pesticide residue analysis"

Analytical Chemistry 89 12 (2017) 6583-6589

3.2.3. Scientific article 5.

"Dual prediction of retention time and collision cross section of emerging contaminants for environmental analyses"

Analytical Chemistry (2021) Submitted

3.3. Discussion of Results

3.1. Introduction

The hyphenation of IMS to HRMS brought several benefits for wide scope screening strategies as highlighted in **scientific articles 1** and **2** from **chapter 2** of this thesis. The drift time alignment, the mass spectral cleaning as well as the additional identification parameter provided by IMS are of special interest for the analysis of OMPs in complex matrices. However, the often elevated complexity of environmental samples pop up different challenges associated to target, suspect and non-target screening analyses such as retention time shifting of target compounds, lack of reference standard information for suspected candidates and/or the necessity of feature prioritization strategies for non-target analysis (Hernández et al., 2019, 2018; Hollender et al., 2017; Menger et al., 2020). Thus, different approaches need to be adopted in order to tackle each of those challenges abovementioned.

In this chapter, attention has been focused on strategies to compensate for retention time deviation of target compounds, and the prediction of analytically-relevant information for suspect candidates for which reference standards are not available.

Approaches to compensate for retention time shifting

Conventional screening strategies based on hybrid systems often rely on the high mass resolving power and mass accuracy attainable by HRMS instruments for compound identification. Positive findings are tentatively identified by comparing their measured and theoretical exact masses, isotope patterns, and fragmentation patterns with that of reference standards or previously reported data in scientific literature (Hernández et al., 2019; Schymanski et al., 2014a). However, the number of potential candidates is often not limited to one, which makes this identification process generally more complicated and time consuming without eliminating the possibility to report false positive findings (Schymanski et al., 2014a).

In the era of seeking for automated strategies for feature identification in wide scope screening analyses, the importance of chromatographic separation seems sometimes overlooked by the excellent performance of HRMS and/or IMS-HRMS (Menger et al., 2020). Thus, the valuable RT information and the optimization of separation capabilities provided by chromatography appear to be a bit disregarded.

Within the compound identification criteria proposed in **scientific article 1** of this thesis, RT is considered as an essential parameter for compound identification at Level 1 and/or useful for levels 2 and 3 considering previously reported data on RT or retention time indices (RTI). Additionally, and to account for RT deviations, Level 1* is proposed provided that IMS-HRMS instrument is used, gathering enough evidence on feature identity by including CCS data. Nevertheless, IMS-HRMS instruments for environmental analysis are not yet widely adopted and implemented in laboratories and, therefore, most of the screening studies still rely on the application of chromatography coupled to hybrid HRMS instruments. Hence, the adoption of strategies to compensate for RT shifting due to matrix interferences should be evaluated.

Corrections for RT deviations have been tackled in different analytical guidelines *e.g.* SANTE guideline (European Commission. Directorate General for Health and Food Safety., 2019) by proposing, mainly, to spike the original sample with the

reference compound of interest and evaluate the RT of the spiked analyte in the sample. However, this requires the availability of reference standards as well as the re-injection of samples. The utilization of RTI may account for RT deviations without the need of re-injecting samples. RTI values are often interpolated by measuring RT of the analyte of interest relative to co-injected standards which are assigned to a fixed RTI value (Rigano et al., 2021). Any shift in RT because of matrix or mobile phase composition is expected to affect the analyte and markers in the same manner so that the RTI remains constant (Hübschmann, 2001). Hence, experimentally found RTI values can be then compared with known values to identify compounds as a system-independent parameter even at an inter-laboratory scale (Rigano et al., 2021).

The applicability of RTI systems has been widely explored in GC where the Kovats retention index normalizes empirical RT to adjacently eluting *n*-alkanes (Babushok, 2015; Strehmel et al., 2008; Ulrich et al., 2013). However, the development of a comprehensive and universal RTI approach for LC is more complex (Babushok, 2015; Rigano et al., 2021). Mobile phase in LC plays a key role in the chemical interactions between analyte and stationary phase; and, therefore, it exceedingly have an influence in the selectivity and retention of a compound (Chatfield and Fitzgerald, 2004). Some studies have been reported dealing with the development of RTI systems for LC. Ulrich *et al.* described the Chromatographic Hydrophobicity Index (CHI) based on the required percentage of organic modifier to elute a certain analyte in a linear gradient (Ulrich et al., 2011). From a different perspective, Bobeldijk *et al.* consider the normalization of retention times using two co-injected standards (Bobeldijk et al., 2002), or a series of n-nitroalkanes by Hall *et al.* (Hall et al., 2012). Major efforts have been devoted within the NORMAN network to develop a comprehensive RTI scaling approach for CECs analysis in LC by using several contaminants for linearly translate RT into RTI values (Aalizadeh, 2021; Dulio et al., 2020).

As it is the case for Kovats index in GC, the co-injection of standards used for indexing is preferred (Rigano et al., 2021). Thus, RTI standards will suffer from the same matrix interferences and, therefore, compensate for RT shifting. However, the addition of standards into real samples may result in the occurring

of false negatives since the corresponding feature will be thought to be caused by the spiked amount of compounds regardless if it was originally present beforehand. Thus, careful interpretation is required as these spiked compounds should not only be associated to RTI, as they can also be present in the sample naturally. Additionally, none of the strategies presented in the literature proposed a system that do not utilize naturally occurring chemicals which can be easily differentiated from sample components.

Retention time and CCS prediction tools for suspect candidates with no reference standard available

There is an increasing trend to move towards screening of environmental samples using large suspect lists of compounds and/or the application of non-target screening strategies to cover many chemical species (Gago-Ferrero et al., 2020; Hernández et al., 2014; Hollender et al., 2019, 2017; Schymanski et al., 2015). Therefore, the outcome can give a better representation of the reality of what is present in the sample since the potential identification of compounds is, in theory, not limited to a restricted list of target chemicals. However, the application of suspect and non-target approaches is often constrained by different factors such as feature prioritization, mass spectral quality or the lack of reference standards required for confirmation (Menger et al., 2020).

The non-availability of reference standards limits the performance of such strategies because no RT, mass spectra and CCS information is available for comparison purposes between standard and empirical observations. Mass spectral libraries such as MassBank are of great help in order to facilitate the putative identification of chemicals by comparing empirical spectra with that previously reported by other research laboratories (Horai et al., 2010). Also, *in silico* fragmentation predictors such as MetFrag can support tentative identification by the comparison of mass spectral data predicted by specialized software (Ruttkies et al., 2016). However, the instrument-dependent nature of RT as well as the still limited availability of online CCS databases requires different strategies to be adopted. In this sense, *in silico* prediction of RT and CCS

values has been explored in the literature. Several studies have been reported for the prediction of RT (Aalizadeh et al., 2019; Bade et al., 2015b, 2015a; Barron and McEneff, 2016; Mollerup et al., 2018; Stanstrup et al., 2015; Yang et al., 2020; Yeung et al., 2020) or CCS values (Bijlsma et al., 2019; Colby et al., 2019; Gonzales et al., 2016; Mollerup et al., 2018; Plante et al., 2019; Ross et al., 2020; Zhou et al., 2017, 2016). Although RT prediction is typically only valid for a laboratory's own chromatographic system, models can be trained and, therefore, adapted to other chromatographic systems (Bijlsma et al., 2019). Contrarily, CCS predictive models can be easily incorporated into other laboratories provided that certain conditions are fulfilled such as IMS technique and/or CCS calibrants used .

The main objective of using predictive tools for RT and CCS is to gather additional confidence in tentative identifications. Commonly, mass spectrometric data is evaluated at first to produce a list of candidate structures that can match with the empirical data. Afterwards, RT and CCS data can be predicted for all listed potential chemical structures and compared with that of the feature. Thus, the different candidate structures will have predicted RT and CCS data that can help to discard some of the structures and select others for further evaluation and/or standard purchase (Bade et al., 2015a, 2015b; Bijlsma et al., 2019). In any case, the incorporation of predicted data into the workflow of data evaluation of suspect and non-target analyses helps to collect more evidence of the potential findings. In the end, reference standards need to be purchased for unambiguous identification, but predictive tools can help to prioritize and reduce costs of buying different reference standards for the same feature.

3.2. Scientific articles

In this chapter, three different studies have been conducted towards the refinement of suspect and non-target screening strategies using conventional HRMS instruments or IMS-HRMS technologies.

The first work (**scientific article 3**) covers the development of a RT interpolation system (namely RTi) to provide robust and valuable information

when using conventional UHPLC-HRMS instruments and analyzing environmental samples. The RTi strategy developed in **scientific article 3** made use of 16 isotopically labeled reference standards as RTi makers, so they can easily be differentiated from naturally occurring compounds in real sample. RTi values can be linearly interpolated using a Kovats-like equation. Alongside, the effect on RTi values of modifications of different chromatographic factors such as mobile phase composition, stationary phase and chromatographic gradient are evaluated. Additionally, the system was built to apply RTi when screening in in both positive and negative ionization modes. Finally, the impact of matrix composition on the performance of the developed RTi approach was evaluated by spiking a set of known standards in 47 different matrices.

The second study (**scientific article 4**) presents an innovative prediction model of CCS values by means of artificial neural networks (ANN). The predictor was trained, optimized and validated using a set of 205 CCS values of small molecules. Predictions of CCS for protonated molecules are done based on eight molecular descriptors. With this model, a prediction accuracy of ± 6% was obtained for the 95% of the cases evaluated with a median error below 2%. This model is applied for different real cases of suspect and non-target screening in spinach samples improving the confidence on tentative identifications of pesticides.

Finally, the third work (**scientific article 5**) presents the development, optimization and validation of a simultaneous prediction tool for RT and CCS of (de)protonated molecules and sodium adducts. In this case, multiple adaptive regression splines (MARS) was used for modelling empirical data, being the first study to explore this approach for RT and CCS modelling. The database presented in **scientific article 1** was used for model development so that a wider range of chemicals than in previous study was covered. The accuracy obtained for the predictions were in the range of ± 2 min for RT, ± 4% for CCS of protonated molecules, ± 5.8% for CCS of deprotonated molecules and ± 5.6% for CCS of sodium adducts. For CCS prediction, the accuracy was improved in comparison to the model presented in **scientific article 4**. The work presented in **scientific article 5**, with RT and CCS predictive models for the same

instrument, strongly facilitates the identification process of organic micropollutants in suspect and non-target strategies.

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

- *Journal of Chromatography A 1568 (2018) 101-107*
- *Analytical Chemistry 89 12 (2017) 6583-6589*
- *Analytical Chemistry (2021) Submitted*

Chapter 3.2.1. Scientific Article 3

DEVELOPMENT OF A RETENTION TIME INTERPOLATION SCALE (RTI) FOR LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY IN BOTH POSITIVE AND NEGATIVE IONIZATION MODES

Alberto Celma, Lubertus Bijlsma, Francisco J. López, and Juan V. Sancho

Journal of Chromatography A 1568 (2018) 101-107

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Development of a Retention Time Interpolation scale (RTi) for liquid chromatography coupled to mass spectrometry in both positive and negative ionization modes

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Highlights

- LC–MS retention time interpolating scale for positive and negative ionization modes.
- 16 isotopically labelled reference standards used as markers for RTi calculation.
- RTi has been demonstrated to remain stable when LC parameters are forced to change.
- The applicability of RTi to samples was demonstrated in 47 distinct matrices.

Keywords

Retention time interpolation scale; Liquid chromatography; High resolution mass spectrometry; Isotopically labelled reference standards; Kovats index; Screening.

Abstract

The accuracy and sensitivity of high resolution mass spectrometry (HRMS) enables the identification of candidate compounds with the use of mass spectrometric databases among other tools. However, retention time (RT) data in identification workflows has been sparingly used since it could be strongly affected by matrix or chromatographic performance. Retention Time Interpolation scaling (RTi) strategies can provide a more robust and valuable

information than RT, gaining more confidence in the identification of candidate compounds in comparison to an analytical standard. Up to our knowledge, no RTi has been developed for LC-HRMS systems providing information when acquiring in either positive or negative ionization modes.

In this work, an RTi strategy was developed by means of the use of 16 isotopically labelled reference standards, which can be spiked into a real sample without resulting in possible false positives or negatives. For testing the RTi performance, a mixture of several reference standards, emulating suspect analytes, were used. RTi values for these compounds were calculated both in solvent and spiked in a real matrix to assess the effect of either chromatographic parameters or matrix in different scenarios. It has been demonstrated that the variation of injection volume, chromatographic gradient and initial percentage of organic solvent injected does not considerably affect RTi calculation. Column aging and solid support of the stationary phase of the column, however, showed strong effects on the elution of several test compounds. Yet, RTi permitted the correction of elution shifts of most compounds. Furthermore, RTi was tested in 47 different matrices from food, biological, animal feeding and environmental origin. The application of RTi in both positive and negative ionization modes showed in general satisfactory results for most matrices studied.

The RTi developed can be used in future LC-HRMS screening analysis giving an additional parameter, which facilitates tedious processing tasks and gain more confidence in the identification of (non)-suspect analytes.

1. Introduction

Mass spectrometry (MS) has revolutionized analytical chemistry. Tandem (MS/MS) instruments are nowadays the most powerful analytical tool widely applied for the qualitative and quantitative determination of organic compounds in complex matrices [1–3], whereas the high quality data obtained by hybrid instruments involving high-resolution mass spectrometry (HRMS) allows rapid, sensitive and selective screening of hundreds of contaminants in for example food [4], environmental [5,6] and forensic samples [7,8], even for compounds for which reference standards are less accessible.

Screening strategies, based on hybrid systems, rely on the high mass resolving power and mass accuracy attainable by HRMS. The data obtained provide relevant information on both (de)protonated molecules and fragment ions, without the need of selecting precursor ions. Positive findings are tentatively identified by comparing their measured exact mass, isotopic pattern and fragmentation pattern to either those of an analytical standard, from scientific literature or theoretically calculated based on the chemical structure [9]. The number of potential candidates is, however, often not limited to one, which makes this identification process generally more complicated and time consuming. Furthermore, reporting false negatives cannot be excluded.

Liquid chromatography (LC) separation, *i.e.* retention time (RT), has not been as routinely incorporated into identification workflows as other *in silico* identification tools and mass spectrometric databases [9,10]. However, chromatographic resolution not only has influence on ion suppression and mass measurement accuracy [11], but RT can also be used as an additional parameter in the identification process and gain more confidence to the obtained results [12,13]. However, RT strongly depends on the type of stationary phase and affinity of compounds with the mobile phase. Other parameters such as flow rate, gradient, column temperature, length and aging, and sample matrix may also strongly affect the retention of compounds [14].

The use of Retention Time Interpolation scale (RTi) evades these variables as it is, in theory, inter-system transferable. The calculation of RTi by measuring RT

relative to co-injected standards (named as markers) can help to overcome the shifting in RT across different situations. Markers are assigned to a fixed RTi value whereas the analyte is interpolated in relation to the markers eluting before and after it. Any shift is expected to affect the analyte and markers in the same manner so that the RTi remains constant [15]. With identification purposes, as it is usually done with RT, experimentally found RTi values can then be compared with known values from analytical reference standards. It is, therefore, more suitable as an identification parameter for wide scope screening strategies. Additionally, RTi could allow extrapolating screening techniques from one laboratory to another.

In gas chromatography (GC), the Kovats retention index, where RT is normalized to the RT of adjacently eluting n-alkanes, is well established and often applied [16–18]. The development of a universal RTi in liquid chromatography (LC), however, is more complicated and has presented many pitfalls [15]. LC is inherently more complex than GC as the mobile phase plays a key role in the chemical interactions with the stationary phase. This influences the selectivity and thus the retention of a compound exceedingly. Some approaches for the calculation of RTi in LC systems have been described in the literature based on the required percentage of organic modifier to elute a certain analyte in a linear gradient (Chromatographic Hydrophobicity Index) [19] or setting the index by means of the partition coefficient (logP) of 10 compounds, mainly pesticides [20]. Other approaches consider the normalization of RT using co-injected standards, either pesticides (KRetI) [21] or a series of *n*-nitroalkanes [22]. KRetI was applied in a non-target analysis for an inter-lab comparison of candidates by means of interpolating a retention index between two co-injected pesticides, chloroxuron and fenuron [21]. However, the series of *n*-nitroalkanes retention index, was developed by injecting the series of compounds before and after the samples and interpolating retention indices using Kovats-like logarithmic equation [22]. In addition, this retention index was based on an isocratic elution only, which is scarcely applied in multi-residue LC methods. As it has been previously explained, the co-injection of standards used for interpolating is preferred. In addition, these standards should easily be differentiated from

compounds naturally occurring in the samples to avoid the reporting of false positives or negatives. Despite that some strategies were applied to LC–MS systems, none of them considered the approach of setting an RTi by means of isotopically labelled reference standards (ILRS). Hence, the aim of this work is to develop an RTi based on ILRS, which *(i)* is robust under different chromatographic conditions, *(ii)* can be applied to any sample matrix, *(iii)* provides an additional identification parameter for screening by LC-ESI-HRMS in both positive and negative ionization modes and *(iv)* is easy to implement in other systems and laboratories.

2. Experimental

2.1. Chemicals and materials

For this study, 121 compounds were used, consisting of 54 isotopically labelled reference standards (ILRS) and 67 analytical reference standards (RS). The complete list of compounds used can be consulted in the **Supplementary information (SI) Table S1**. ILRS and RS were purchased from Across Organics (Geel, Belgium), Aventis Pharma (Madrid, Spain), Bayer Hispania (Barcelona, Spain), Cayman Chemicals (Ann Arbor, MI, USA), CDN Isotopes (Quebec, Canada), Cerilliant (Round Rock, TX, USA), Dr. Ehrenstorfer (Augsburg, Germany), Fluka (Buchs, Switzerland), Fort Dodge Veterinaria (Gerona, Spain), National Measurement Institute (Pymble, Australia), Riedel-de Haën (Seelze, Germany), Sigma–Aldrich (St Louis, MO, USA), Toronto Research Chemicals (Ontario, Canada), Vetoquinol Industrial (Madrid, Spain) and Witega (Berlina, Germany). All reference standards had purities higher than 98% *(w/w)*. Leucine enkephalin, used for mass correction, was purchased from Sigma–Aldrich (St. Louis, MO, USA).

HPLC-grade methanol (MeOH), HPLC-grade acetonitrile (ACN) and formic acid (HCOOH, >98%) were supplied by Scharlau (Barcelona, Spain). HPLC-grade water was obtained by purifying demineralized water in a Milli-Q plus system from Millipore (Bedpore, MA, USA). A standard stock solution of each compound was prepared at a concentration level between 1000 μg L^{-1} and 10 g L^{-1} in MeOH or ACN.

2.2. Selection of markers

A preliminary study was performed with each of the ILRS included in the study to establish RT, peak intensity and in-matrix reproducibility. The final selection of markers for RTi calculation was based on different criteria. First of all, the RTi strategy needs to be extended to the whole chromatographic run and marker distribution should cover from the very first compound to the last one in an arrangement as equally distributed as possible. The proportionality in the distribution of markers across the chromatogram is important in terms of RTi

values comparison. Second, regulated compounds such as drugs of abuse or new psychoactive substances were whenever possible avoided as well as those of higher cost. Third, compound ionization efficiency was considered for the establishment of an estimated concentration required for good peak intensity in complex matrices. In summary, full proportional spectrum coverage, in-matrix reproducibility, peak intensity, compound family and costs were considered for the selection of appropriate compounds.

Additional information regarding both markers and analytes can be found in the **Supplementary information Table S2**.

2.3. Testing matrices

A complete list of matrices used in the study (food, environmental samples, animal feed and biological fluids) and the sample treatment applied are available in SI (**Table S3** and **Table S4 in Supplementary information**). The corresponding extracts were spiked with both the set of markers (ILRS) and reference standards (used as target analytes). A suspect screening analysis was performed in order to obtain RTi values in real samples.

2.4. Instrumentation

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was coupled to a quadrupole TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), with a Z- spray- ESI interface operating in positive and negative ion mode. An Acquity UPLC BEH C18 analytical column 2.1 × 100 mm with 1.7 μm particle size (Waters) and a Cortecs C18 2.1 × 100 mm with 2.7 μm particle size were employed for chromatographic separation. Mobile phase, at a flow rate of 0.3 mL min^{-1}, consisted of water and MeOH both with 0.01% HCOOH. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 10% B; 14 min. 90% B; 16 min. 90% B; 16.01 min, 10% B; 18 min, 10% B. The column was set at 40 °C. MS data were acquired in the range of *m/z* 50–1000. A capillary voltage of 0.7 kV in positive mode and 2.5 kV in negative mode were used with a cone voltage of 25 V. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 650 °C and the source temperature at 120 °C. For automated accurate mass measurement, the lock-

spray probe was used, using a lock mass solution of leucine enkephalin (2.5 mg L–*1*) in ACN:water (1:1 *v/v*) at 0.1% HCOOH pumped at 30 µL min^{-1} through the lock-spray needle. The (de)protonated molecule of leucine enkephalin at *m/z* 556.27658 in positive mode and *m/z* 554.26202 in negative mode was used for recalibrating the mass axis and ensuring a robust accurate mass measurement along time.

For MSE, two acquisition functions with different collision energies were generated. The low collision energy function (LE) with a collision energy of 4 eV, and the high collision energy function (HE) with a collision energy ramping from 15 to 40 eV. MS data were acquired in continuum mode and processed with the screening platform within UNIFI v1.8 (Waters Corporation).

2.5. Retention Time Interpolation scale (RTi) calculation

Based on the equation developed by Kovats [23] and the modifications applied by Van der Dool and Kratz [24], the equation used for the calculation of RTi values is shown in **Eq. (1)** where *n* corresponds to the elution position of the marker eluting just before the analyte (j) and *n* +1 corresponds to the markers eluting right after the analyte. In addition, the deviation was calculated as a relative difference between the measured value and an average RTi value (obtained with the injection of a mixture of standards in solvent). The equation used is shown in **Eq. (2).**

$$RTi_j = 100 \cdot \left(n + \frac{\left(t_R'\right)_j - \left(t_R'\right)_n}{\left(t_R'\right)_{n+1} - \left(t_R'\right)_n} \right) \quad (1)$$

$$Dev_j(\%) = 100 \cdot \frac{(RTi)_j - \overline{RTi}_{Solvent}}{\overline{RTi}_{Solvent}} \quad (2)$$

2.6. General data processing

The impact of chromatographic parameters in RTi strategy were tested by spiking both the set of markers and RS in solvent. Standard RTi ($RTi_{Solvent}$) values were established by calculating them when injected with the conditions specified

in Section 2.4. Then, experimentally obtained values after forcing chromatographic parameters were compared with RTi in solvent by means of **Eq. (2)**.

The same strategy is applied for assessing the impact of matrix. Both markers and analytes were spiked in sample extracts and deviations of experimental RTi values were calculated by means of **Eq. (2)**.

The maximum allowed absolute RT deviation in several guidelines is ≤0.1 min, which represents 5% of the average 2 min window between markers in RTi-P. Therefore, RTi deviation should be below 5% as a way of translating absolute deviation from guidelines to our RTi system.

3. Results and discussion

3.1. Selection of markers

The first selection of potential markers to include in the study was based on their availability at our lab as well as their amenability for liquid chromatography. A set of 54 different ILRS was selected and evaluated as markers for its application in both positive and negative ionization modes. The development and optimization of RTi strategy was performed separately for each ionization mode. It was decided to use ILRS as markers because of the applicability of this strategy in screening analyses. The coupling of LC with HRMS allows the differentiation of naturally occurring compounds in a matrix from the isotopically labelled ones used as markers. Therefore, the potential reporting of false positives and negatives was avoided since compounds intentionally spiked to a sample can easily be differentiated from the naturally occurring ones by their m/z.

The final set of markers selected encompassed 12 markers for RTi in positive ionization mode (RTi-P) and 6 for negative ionization mode (RTi-N) (**Figure 1**). The markers showed good performance in both solvent and matrix-matched analysis, except for ecgonine-d3 which was observed in around 80% of the matrices as it elutes early in the chromatogram and can be more affected by matrix suppression.

Finally, markers were arbitrarily assigned a value of *n* depending on the elution order ranging from *n* = 0 (ecgonine-d3) to *n* = 11 (diethylhexylphathalate-d4) in RTi-P and *n* = 0 (ecgonine-d3) to *n* = 5 (THC-COOH-d3) in RTi-N (**Table 1**). When applying **Eq. (1)**, RTi values ranged from 0 to 1100 in RTi-P and from 0 to 500 in RTi-N.

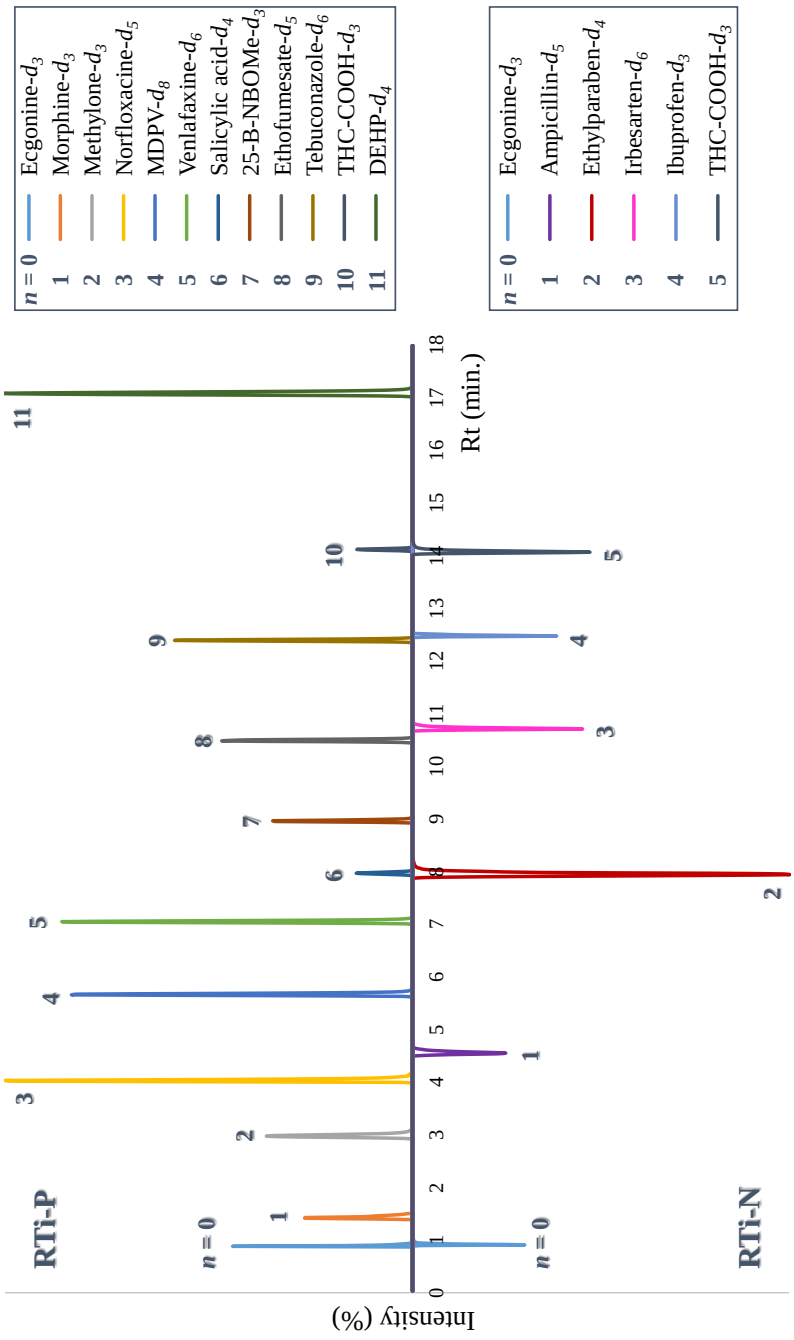

Figure 1. Marker distribution across chromatographic injection for RTi-P (top) and RTi-N (bottom).

Table 1. Set of markers for RTi in both positive and negative modes.

		RTi-P		RTi-N	
n	**RTi value**	**Compound**	**RT (min)**	**Compound**	**RT (min)**
0	0	Ecgonine-d_3	0.98	Ecgonine-d_3	0.98
1	100	Morphine-d_3	1.43	Ampicillin-d_5	4.60
2	200	Methylone-d_3	2.97	Ethylparaben-d_4	8.00
3	300	Norfloxacine-d_5	4.03	Irbesartan-d_6	10.77
4	400	MDPV-d_8 [a]	5.66	Ibuprofen-d_3	12.54
5	500	Venlafaxine-d_6	7.02	THC-COOH-d_3	14.12
6	600	Salicylic acid-d_4	7.97		
7	700	25-B-NBOMe-d_3 [b]	9.03		
8	800	Ethofumesate-d_5	10.48		
9	900	Tebuconazole-d_6	12.39		
10	1000	THC-COOH-d_3 [c]	14.12		
11	1100	DEHP-d_4 [d]	17.09		

[a] MDPV-d_8: methylendioxypyrovalerone-d_8

[b] 25-B-NBOMe-d_3: 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine-d_3

[c] THC-COOH-d_3: 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol-d_3

[d] DEHP-d_4: di(2-ethylhexyl)phthalate-d_4

3.2. The impact of chromatographic parameters

Changes in chromatographic conditions should not affect the RTi calculation since markers should, in theory, correct for possible shifts in RT. The performance of RTi approach was evaluated forcing several chromatographic parameters *i.e.* variation of injection volume, gradient, column aging and type of solid support of stationary phase in chromatographic column. In-solvent mixtures of markers and reference standards (acting as target analytes) were injected under these different conditions. Analytes of a wide range of polarity were chosen in order to cover the whole chromatogram and to have analytes within each interval between two markers. RTi values of analytes in the different

conditions were compared with those obtained with the conditions explained in Section 2.4 to assess the impact of the different chromatographic parameters.

Injection volumes 10, 20, 30, 40 and 50 µl (n = 5) were tested resulting in little distortion of RTi. The majority of the compounds (85% for RTi-P and 100% for RTi-N) showed a deviation in their RTi < 5%. In theory, if the organic phase of the sample solvent is the same as the initial conditions of the gradient, a higher injection volume should not alter the eluent composition. In general, if detection of low concentrated compounds is required, injection volume can be increased without affecting the performance of RTi considerably.

Retention capability of a chromatographic column is often reduced with the column aging. For the development of RTi-P, the performance of a new chromatographic column was compared to the performance after roughly 800 injections. Although many injections can still be done with the column, RT varied in the range of 0–1 min with an average variation of 0.15 min. RTi values showed a deviation <5% and >10% in 55% and 27.5% of the cases, respectively. The high deviation observed *i.e.* 45% of compounds above 5% deviation remarked that the degradation of the stationary phase in the column produced different retention patterns between analytes and markers. Additionally, for RTi-N, the effect of column aging was assessed after 300 injections (from injection 700 to injection 1000). In this case, values showed a deviation of <5% in 90% of the cases (10% for >10% deviation). These results considerably differ from the ones obtained for RTi-P. The different percentage of compounds having a deviation value >10% also highlighted that the degradation of the column is a progressive effect.

In terms of gradient, the effect of 5, 10, 15 and 20% of organic phase at the beginning of the gradient was tested (n = 5). Compounds eluting at the beginning of the chromatogram were strongly affected suffering from variations in the RT of up to 2.3 min. However, RTi values showed a deviation <5% in 72% of the cases (RTi-P) and 71% of the cases (RTi-N). Only 7% of the compounds showed a deviation >10% for RTi-P and 14% for RTi-N. Additionally, the impact of extending or shortening the chromatographic elution was assessed by the

comparison of some run durations (5, 10, 15, 20, 25 and 30 min.). In total, 73% of the compounds showed a deviation in their RTi-P <5% (86% of the cases in RTi-N) and only 5% of the compounds had a deviation >10% for both RTi-P and RTi-N.

The effect of implementing the same strategy by using a different type of column was assessed. The utilization of a chromatographic column of a completely different stationary phase would completely change the chromatographic retention mechanisms and, therefore, makes the application of the RTi strategy not feasible. Therefore, some C18 columns were tested, but with different solid support for the stationary phase which, in theory, would not strongly affect the retention of compounds.

A Cortecs C18 and Acquity UPLC BEH C18 columns were compared. BEH columns are polymeric based columns with a particle size of 1.7 µm, whereas Cortecs are made with 2.7 µm solid-core silica particles. Despite both chromatographic columns were reverse phase, the distinct solid support and particle size was expected to produce small variations in absolute RT from one column to the other. When using Cortecs column, 59% of the compounds showed a deviation on their RTi-P value <5% (73% for RTi-N). Notwithstanding, 24% of compounds showed a deviation >10% (18% for RTi-N). A clear trend was observed differentiating two regions in the chromatogram. From 0 to 10 min, where high RTi value deviations were observed, and from 10 to 18 min where RT correction by means of RTi was satisfactory. Those deviations could be due to the selectivity of the stationary phase *i.e.* the distinct solid support. Therefore, alternative markers were studied in order to improve performance in this type of column in the first half of the chromatographic run. However, no improvements were observed and it was decided to maintain the set of markers optimized in previous sections. These deviations suggested that the RTi strategy could not be directly implemented in a different type of column than the one used for its development without previous adaptive studies.

Table 2 shows a summary of the deviations in RTi values for analytes in both RTi-P and RTi-N when some chromatographic parameters were forced to

change. As previously stated, the application of UHPLC-HRMS techniques for screening analyses usually means that resolution power is only entrusted to mass analyzer and therefore, the chromatography is rarely modified to improve compound separation. Even though, RTi application allows, as demonstrated, the variation of the some chromatographic parameters such as injection volume and gradient if better separation of compounds or signal are necessary without affecting the interpolating performance. Additionally, the RTi strategy can be applied regardless the amount of organic compound present in the extract because any possible distortion of RT is be corrected by RTi.

Table 2. Average percentage of analytes showing RTi deviations below 2.5% and 5%, and above 10% when modifying different chromatographic parameters for both RTi-P and RTi-N.

	Deviation in RTi-P			**Deviation in RTi-N**		
	< 2.5%	**< 5%**	**> 10%**	**< 2.5%**	**< 5%**	**> 10%**
Injection Volume Variation	63%	85%	2%	97%	100 %	0%
Percentage of organic at the beginning of gradient	42%	72%	7%	62%	71%	14%
Chromatographic gradient modification	45%	73%	5%	64%	86%	5%
Column aging	36%	55%	28%	90%	90%	10%
Type of solid support for stationary phase	50%	59%	24%	73%	73%	18%

3.3. The influence of matrix

The influence of different matrices on the RTi performance was evaluated by assessing the reproducibility of RTi values for RS in 47 different matrices. The selection of the matrices was based on the availability of extracts, the availability of raw matrix (in the cases that sample treatment was simple), and the coverage of different scientific fields such as food (including acidic, basic, fatty and sugary matrices), environmental (drinking, surface and ground water as well as influent and effluent wastewater), feed (bovine, poultry and rabbit feed) or biological

fluids (plasma, blood and urine) to demonstrate its applicability at different research areas. The impact of the matrix on the calculation of RTi was evaluated by spiking the set of 16 markers and 67 analytical reference standards to 47 samples/extracts of different origin. A target screening was performed in order to obtain the RT and RTi for all compounds. **Table S5 (in Supplementary information)** summarizes RT and RTi for all markers and analytes in all matrices tested for both RTi-P and RTi-N. For comparison purposes, in-matrix RTi deviation from RTi in solvent were calculated resulting in average values below 5% in the 93% of the cases for RTi-P and only 2% of the cases analyzed showed a deviation greater than 10% from the RTi values in solvent. For the analysis of RTi-N, the five most troublesome matrices and other five random matrices were analyzed and the same behavior was observed.

The current analytical guidelines for compound identification generally require an absolute deviation in RT from the correspondent standard less than 0.1 min [25–27]. When applying this criteria, identification would not have been possible for the 7% of the RS spiked in matrices, since analytes differ (>0.1 min) in RT compared to its standard in solvent. **Table 3** shows some examples of potential false negatives in matrices. Nevertheless, these could all be corrected by the application of RTi-P. An illustrative example is demonstrated in **Figure 2**, where azithromycin in lettuce showed a RT deviation of 0.38 min. When strictly applying the guidelines, azithromycin would not have been reported *i.e.* resulting in a false negative. However, its RTi differed only 0.63% with the RTi of the standard (well below the 5% deviation threshold) and would, therefore, not have been discarded.

As demonstrated, the application of RTi is feasible in a wide range of matrices. Moreover, its application in a real screening scenario would be much more successful since it is known that usually the majority of compounds did not elute in the first part of the chromatogram [28,29], which is the region of not optimal performance of RTi. So, the application of RTi in a real suspect screening would lead to even higher percentages of success than those obtained when the whole chromatogram was covered with analytes for RTi development.

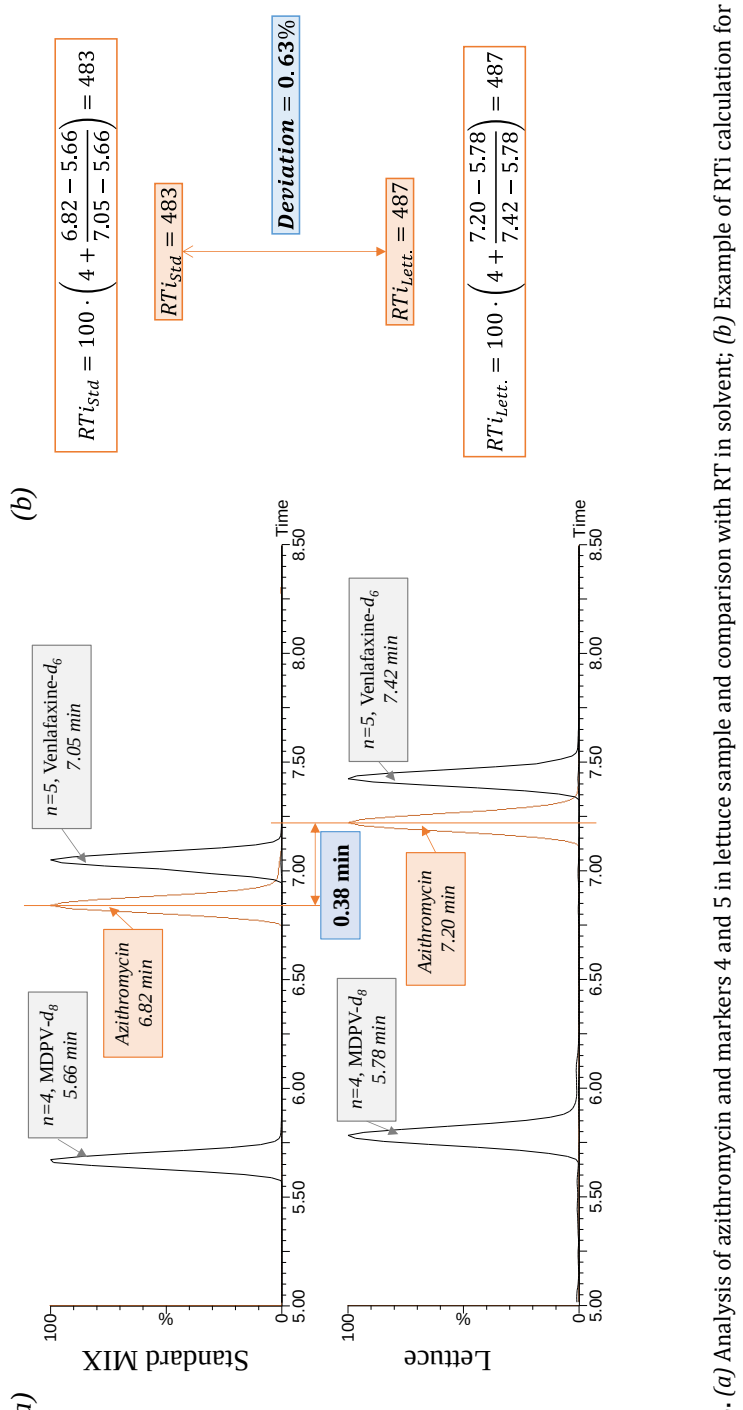

Figure 2. *(a)* Analysis of azithromycin and markers 4 and 5 in lettuce sample and comparison with RT in solvent; *(b)* Example of RTi calculation for azithromycin.

Table 3. Examples of potential false negatives in matrix analysis corrected by RTi-P application.

Compound	Matrix	Retention time (min)			Retention Time Interpolation scale (RTi-P)				
		Std.	Matrix	Dev. (min)	Std.		Matrix		Dev. (%)
Amphetamine	White Bread	3.42	3.53	0.11	n=2; 2.97 min 3.42 min n=3; 4.03 min	243	n=2; 3.04 min 3.53 min n=3; 4.07 min	248	2.04 %
Azithromycin	Lettuce	6.82	7.20	0.32	n=4; 5.66 min 6.82 min n=5; 7.05 min	483	n=4; 5.78 min 7.20 min n=5; 7.42 min	487	0.63 %
Clenbuterol	Industrial Bakery	4.96	5.06	0.10	n=3; 4.03 min 4.96 min n=4; 5.66 min	357	n=3; 4.06 min 5.06 min n=4; 5.77 min	358	0.33 %
Thiamethoxam	Coffee	3.80	3.68	-0.12	n=2; 2.97 min 3.80 min n=3; 4.03 min	279	n=2; 2.97 min 3.68 min n=3; 4.05 min	266	-4.61 %
Sarafloxacin	Influent WW	4.79	5.06	0.27	n=3; 4.03 min 4.79 min n=4; 5.66 min	346	n=3; 4.16 min 5.06 min n=4; 5.88 min	352	1.71 %

In addition, wide-scope screening strategies are usually affected by the wide range of matrices analyzed as well as a combination of other chromatographic parameters alterations (column aging, percentage of organic phase in extract…). Oftentimes, the analyst faces cases where exact mass of (de)protonated molecule and fragment ions, and isotopic pattern fit within a candidate but RT is deviated from the standard. Consequently, further analysis by spiking the extract with the candidate compound or updating RT databases should be performed, resulting in time-consuming and costly tasks. For broader applicability of these type of strategies, the utilization of RTi will avoid these 'extra' analyses, also reducing the rate of false negatives in wide-scope screening analyses.

4. Conclusions

A robust retention time interpolation scale strategy has been developed for screening applications in UHPLC-HRMS systems in both positive and negative ionization modes by means of 16 ILRS. The impact of several chromatographic parameters on its performance has been tested resulting in satisfactory performance. Consequently, RTi strategy allows the modification of some chromatographic parameters if better resolution of compounds is required. In addition, the strategy has been tested for its application through nearly 50 matrices from different origin (*i.e.* food, biological, animal feeding and environmental), showing a success rate of approximately 90% in the identification of analytes in both positive and negative ionization modes. RTi developed has shown an additional value for LC-HRMS screening applications laying the basis of a harmonized retention parameter that could easily be implemented in other systems and laboratories. The substitution of any marker with another ILRS could be evaluated for further application if any ILRS used in this study is not available in the implementing laboratory. Lately, the in-lab RTi values for the reference standards of interest need to be established for specific chromatographic conditions.

Acknowledgements

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Supplementary data

In this section, five tables are included to have supportive information on the written text. Tables S1 shows the complete list of chemicals used in this study for their potential application as RTi markers and also the analytes used to assess the performance of the system. Table S2 indicates chemical information about the compounds finally used in the approach. Table S3 and S4 show the different matrices assessed during method development as well as the sample treatment applied. Finally, Table S5 indicates the complete dataset of RT and RTi values for both markers and analytes in the tested matrices.

Supplementary data to this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2018.07.030 and in this chapter after the section “References”. Due to space limitations, Table S2 and S5 can only be found in the online version.

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Supplementary data

Table S1: Isotopically labelled reference standards (ILRS) and Reference Standards (RS) used in this study.

Isotopically Labelled Reference Standards (ILRS)		
(3,4-Methylenedioxy)methamphetamine-d_5 (MDMA-d_5)	Codeine-d_6	Methylone-d_3
N-Ethyl-3,4-methylenedioxyamphetamine-d_5 (MDEA-d_5)	Di(ethylhexyl)phathalate-d_4 (DEHP-d_4)	Morphine-d_3
11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol-d_3 (THC-COOH-d_3)	Dichloroaniline-d_2	Naphyrone-d_5
25-B-NBOMe-d_3	Diclofenac-d_4	Norfloxacine-d_5
25-C-NBOMe-d_3	Diuron-d_6	Olanzapine-d_3
25-I-NBOMe-d_3	Ecgonine-d_3	Omeprazole-d_3
3,4-Methylenedioxyamphetamine-d_5 (MDA-d_5)	Ethofumesate-d_5	Oxamyl-d_3
6-Monoacetylmorphine-d_6	Ethylparaben-d_4	Paroxetine-d_4
Acetaminophen-d_4	Fenitrothion-d_6	Phosmet-d_6
Amphetamine-d_6	Heroin-d_9	Pyrene-d_{10}
Ampicillin-d_5	Ibuprofen-d_3	Quizalofop-*p*-ethyl-d_3
Atorvastatin-d_5	Iprodione-d_7	Salicylic Acid-d_4
Benzoylecgonine-d_3	Irbesartan-d_6	Sarafloxacine-d_8
Butylone-d_3	Ketamine-d_4	Simvastatin-d_6
Carbamazepine 10,11 epoxide -d_{10} (C10,11E-d_{10})	Maleic hidrazide-d_2	Tebuconazole-d_6 (TEB-d_6)
Clorpyriphos-d_{10}	MCPA-d_3	Terbuthylazine-d_5
Cocaethylene-d_8	Methamphetamine-d_5	Valsartan-d_8
Cocaine-d_3	Methylenedioxypyrovalerone -d_8 (MDPV-d_8)	Venlafaxine-d_6

Table S1 (cont.): Isotopically labelled reference standards (ILRS) and Reference Standards (RS) used in this study.

Reference Standards (RS)		
Aldicarb	Fenhexamid	Olanzapine
Amoxicillin	Fenthion	Oxadixyl
Amphetamine	Flubendazole	Oxolinic acid
Amprolium	Flumequine	Parathion-methyl
Atorvastatin	Fluroxypir	Pefloxacin
Azythromycin	Furaltadone	Pendimetaline
Bentazone	Furosemide	Pipemidic acid
Bixafen	Gemfibrozil	Pravastatin
Boscalid	Hexaflumuron	Pymethrozine
Bromophos ethyl (BET)	Ibuprofen	Salbutamol
Carbamazepine	Imidacloprid	Salicylic acid
Carbaryl	Iprodione	Sarafloxacin
Chloridazon	Irbesartan	Sulfadoxine
Ciprofloxacin	Lincoycin	Sulfamethazine
Clarythromycin	Linuron	Tetracycline
Clenbuterol	Lorazepam	Thiacloprid
Deethylatrazine	Lufenuron	Thiamethoxam
Diclofenac	Mepanipyrim	Thiobencarb
Diflufenican	Methabenzthiazuron (MTBZ)	Thiophanate-methyl (TPM)
Dimethoate	Methcathinone	Triadimefon
Enalapril	Methiocarb	τ-fluvalinate
Enrofloxacin	Moxifloxacine	
Ethoxyquin dimer (ETD)	Nalidixic acid	

Table S3: Matrices used to evaluate the influence on RTi performance.

Matrix	**Code**	**Matrix**	**Code**
Apple and pear	(Diet MP)	Whole meal bread	(Diet PI)
Biscuits	(Diet GM)	Wine	(Diet V)
Breakfast cereals	(Diet CD)	Yoghurt [a]	(Diet Y)
Cheese	(Diet Q)	Drinking water	(DW)
Coffee [a]	(Diet C)	Effluent WW	(EWW)
Custard and smoothies	(Diet NB)	Ground water	(GW)
Dried fruits and nuts	(Diet FS)	Surface water [a]	(SW)
Eggs	(Diet HU)	Bovine feed [a]	(Feed BO)
Grapes	(Diet UV)	Poultry feed	(Feed PO)
Industrial bakery	(Diet BI)	Rabbit feed	(Feed RA)
Lettuce	(Diet LE)	Herbal blends	(HB)
Milk	(Diet L)	Plasma	(Plasma)
Olive oil	(Diet AO)	Serum	(Serum)
Olives and pickles	(Diet AE)	Urine 1 [a]	(Urine 1)
Onion [a]	(Diet O)	Urine 2	(Urine 2)
Orange juice	(Diet ZN)	Sludge, aqueous phase	(Sludge aq.)
Pasta	(Diet PA)	Sludge, solid phase	(Sludge sol.)
Peach, pear & grapes juice [a]	(Diet ZMPU)	Influent WW 1	(IWW 1)
Rice	(Diet AR)	Influent WW 2	(IWW 2)
Sliced bread	(Diet PM)	Influent WW 3	(IWW 3)
Soy products	(Diet PS)	Influent WW 4	(IWW 4)
Sunflower oil	(Diet AG)	Influent WW undiluted 1 [a]	(IWW und. 1)
Tomato [a]	(Diet TO)	Influent WW undiluted 2	(IWW und. 2)
White bread [a]	(Diet PB)		

[a] Used in both RTi-P and RTi-N.

Table S4: Sample treatment of matrices included in this study.

Group	Matrix	Sample treatment	Concentration factor	Organic Solvent (%)	Ref.
Group 1	Diet PB	Extr.: Acetonitrile:water (80:20) 0.1% HCOOH; Centrif. 4000 rpm 10 min; Dil. of supernatant	Final content: 0.0625 g sample ml-1	ACN (5%)	(1)
	Diet PM				
	Diet PI				
	Diet PA				
	Diet AR				
	Diet FS				
	Diet GM				
	Diet BI				
	Diet CD				
Group 2	Diet AE	Extr.: Acetonitrile:water (80:20) 0.1% HCOOH; filtration; Dil.	Final content: 0.05 g sample ml-1	ACN (<80%)	(1)
	Diet MP				
	Diet HU				
	Diet L				
	Diet Y				
	Diet NB				
	Diet PS				
	Diet Q				
	Diet UV				
Group 3	Diet C	Dil. with 0.1 % HCOOH HPLC water; Centrif. 4000 rpm 15 min	Final content: 0.1 g sample ml-1	-	(1)
	Diet V				
	Diet ZN				
	Diet ZMPU				
Group 4	Diet AO	ACN LLE; Dil.	Final content: 0.0625 g sample ml-1	ACN (25%)	(1)
	Diet AG				
Group 5	Diet LE	QuEChERS	-	MeOH (10%)	*
	Diet TO				
	Diet O				
Group 6	DW	Centrif. 4500 rpm 5 min	-	-	(2)
	EWW				
	GW				
	SW				
Group 7	Feed BO	Extr.: ACN 1% HCOOH; Centrif.; Dil.	x 1/10	ACN (10%)	(3)
	Feed PO				
	Feed RA				
Group 8	HB	Extr.: Acetone; Dil.	Final content: 0.003 g sample ml-1	Acetone (10%)	(4)
Group 9	Plasma	Deproteinization with ACN; Centrif.	x 1/4	MeOH (10%)	(5)
	Serum				
Group 10	Urine 1	Centrif. 12000 rpm 10 min; Dil.	x 1/2	-	(6)
	Urine 2				
Group 11	Sludge aq.	HLB SPE; Centrif.	x 50	MeOH (10%)	(7)

Group	Matrix	Sample treatment	Concentration factor	Organic Solvent (%)	Ref.
Group 12	Sludge sol.	Extr.: MeOH:water (1:1) 0.5% HCOOH; Centrif.; (x2)	Final content: 0.1 g sample ml-1	MeOH (10%)	(7)
Group 13	IWW 1	HLB SPE	x 25	MeOH (10%)	(8)
	IWW 2				
	IWW 3				
	IWW 4				
Group 14	IWW und. 1	Direct injection	-		-
	IWW und. 2				

*: Not yet published but validated methodology followed by researchers at Research Institute for Pesticides and Water, Universitat Jaume I, Castellón.

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Chapter 3.2.2. Scientific Article 4

PREDICTION OF COLLISION CROSS-SECTION VALUES FOR SMALL MOLECULES: APPLICATION TO PESTICIDE RESIDUE ANALYSIS

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Prediction of Collision Cross-Section Values for Small Molecules: Application to Pesticide Residue Analysis

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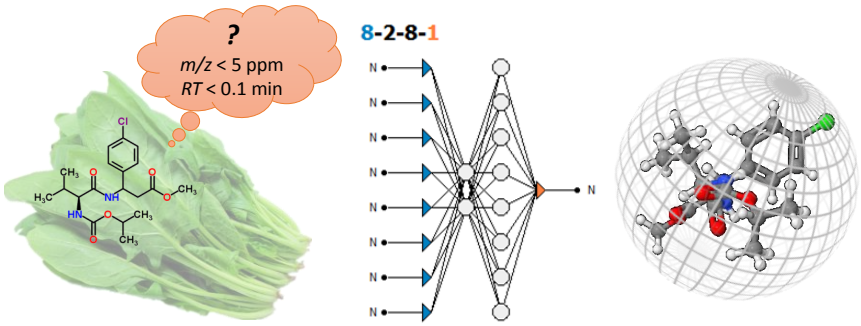
Graphical Abstract



Abstract

The use of collision cross-section (CCS) values obtained by ion mobility high-resolution mass spectrometry has added a third dimension (alongside retention time and exact mass) to aid in the identification of compounds. However, its utility is limited by the number of experimental CCS values currently available. This work demonstrates the potential of artificial neural networks (ANNs) for the prediction of CCS values of pesticides. The predictor, based on eight software-chosen molecular descriptors, was optimized using CCS values of 205 small molecules and validated using a set of 131 pesticides. The relative error was within 6% for 95% of all CCS values for protonated molecules, resulting in a median relative error less than 2%. In order to demonstrate the potential of CCS prediction, the strategy was applied to spinach samples. It notably improved the confidence in the tentative identification of suspect and nontarget pesticides.

1. Introduction

Ion mobility mass spectrometry (IMS) separates ions on the basis of their shape, mass, size/charge ratios as well as their interactions with a buffer gas, and it is a powerful analytical tool for investigating complex samples [1–7]. Collisions occur between ions and a neutral buffer gas under an electric field, resulting in different drift times in the range of milliseconds. The collision cross-section (CCS) of an ion is a value unique to IMS and is derived from the drift time. The chemical structure and three-dimensional conformation of a molecule influences the CCS, with smaller (more spherical) molecules having smaller CCS values than more extended ones (planar structures, extended chains, helices) [8]. With all of this chemical information within one value, CCS can thus be used as a useful additional parameter in the identification of a compound.

Traditionally, detection and identification of compounds are based on the comparison of retention time and accurate mass with a reference standard [9]. When a reference standard is unavailable, alternative means are necessary to provide "tentative identification" such as retention time prediction [10–14] and searching in mass spectral libraries [15–19]. However, these databases are far from all-encompassing, leading to theoretical mass spectra being included in various databases, which have their own limitations [20]. Retention time is difficult to predict accurately because of the inherent repeatability problems associated with the matrices. Moreover, retention time prediction is typically only valid for a laboratory's own chromatographic system and therefore not easily transferable.

One further problem with conventional liquid chromatography (LC)-based approaches are that isobaric and isomeric components from the sample matrix can interfere with the signal from a residue present in the sample or be mistaken for residues not present in the sample, giving rise to false negatives or positives, respectively. CCS values, meanwhile, are undertaken in the gas phase, remotely from the ion source, meaning they are unaffected by the sample matrix and are consistent between instruments and across a range of experimental conditions [21]. For these reasons, the use of CCS values as a three dimension (alongside

retention time and exact mass) to identify compounds presents a great avenue for analytical chemists.

Although experimental CCS values have been proven invaluable in identifying various compounds, there is currently no empirical CCS library, so any suspect or nontarget compounds will not be able to be identified or confirmed on the basis of CCS. For this reason, prediction of CCS has become an area of great interest with various computational means being used, predominantly for small molecules [3,22] and peptides [23,24]. These calculations were based on the molecular modelling simulation of ions and the inert gas, but they needed immense computational power and time to calculate even for a few small molecules, giving estimated relative errors between 3 and 30% of experimental values [2,4,25].

In order to shorten the calculation times, there has been a shift toward machine-based learning tools, such as artificial neural networks (ANNs) and support vector machines (SVMs). These approaches reverse the approach of conventional molecular modelling, with empirical data used in the first step for the prediction of drift time [26] or CCS [27], with up to 90% of all compounds having an error less than 10%.

Globally, pesticide residue analysis in food has high interest [28–31]. The great number of pesticides potentially present in a food sample makes the use of efficient tools necessary to facilitate the identification of compounds that do not form part of the target compounds included in the method. The combination of IMS with a potent prediction of CCS will facilitate the screening of a large number of suspect and nontarget compounds, including metabolites, improving the reliability of tentative identifications. Therefore, the aims of this work are 2-fold: *(i)* to develop an accurate ANN-based tool for the prediction of CCS values of small molecules, based on empirical CCS values of 205 protonated compounds (**Table S1**, **Figure S1**), *(ii)* to demonstrate the "proof of concept" and the potential for future applications by applying the prediction model to blind spinach samples in order to gain confidence in the identification of pesticides for which no experimental CCS values and/or reference standards are available.

2. Experimental Section

2.1. Instrumentation

A Waters Acquity I-Class UPLC system (Waters, Milford, MA, U.S.A.) was interfaced to a VION IMS-QTOF mass spectrometer, using an electrospray ionization (ESI) interface operating in positive mode.

The chromatographic separation was performed using an Acquity UPLC BEH C18 2.1 × 100 mm, 1.7 µm (Waters) at a flow rate of 450 µL/min. Gradient elution was performed using mobile phases of A = H_2O and B = MeOH, both with 10 mM ammonium acetate (pH 5.0). The initial percentage of B was 2%, which was linearly increased to 99% after 25 s for 12 min, followed by a 35 s isocratic period, then, returned to initial conditions over 4 min. The total run time was 17 min. Nitrogen was used as the drying gas and nebulizing gas.

A capillary voltage of 0.8 kV and cone voltage of 20 V were used. The desolvation temperature was set to 550 °C, and the source temperature to 120 °C with a source offset of 80 °C. The cone gas flow was 50 L/h and desolvation gas flow of 1000 L/h. The column temperature was set to 45 °C and sample temperature at 4 °C. MS data was acquired using the VION in high-definition (HD)MS^E mode, in the range 50–1200 *m/z*, with N_2 as the drift gas, an IMS wave velocity of 250 m/s and wave height ramp of 20–50 V. Two independent scans with different collision energies were acquired during the run: a collision energy of 4 eV for low energy (LE) and a ramp of 15–40 eV for high energy (HE). $HDMS^E$ implies drift time alignment between LE and HE spectra keeping only fragment ions related to parent ions [5]. The LE and HE functions settings were for both a scan time of 0.25 s. Argon (≥99.999%) was used as collision-induced dissociation (CID) gas. All data was examined using an accurate mass screening workflow within UNIFI informatics platform from Waters Corporation.

2.2. Sample Treatment

Blind spinach samples (n = 3) were prepared by spiking at 0.05 mg/kg with 10 pesticides that were unknown to the analyst (**Table S2**). Sample treatment was performed with the QuEChERS method [32,33]. Briefly, a 15 g homogenized

spinach sample was weighed into a 50 mL centrifuge tube to which 15 mL of acetonitrile containing 1% acetic acid was added along with 6 g of magnesium sulfate and 1.5 g of sodium acetate, and the tube was shaken and centrifuged. A portion of the extract was mixed with 3:1 (w/w) magnesium sulfate/primary secondary amine sorbent (200 mg/mL extract) and centrifuged. Analyses were performed by injecting 5 µL of the final extract into the VION system.

2.3. CCS Prediction using Artificial Neural Networks

The artificial neural network (ANN) CCS predictions were made using Alyuda NeuroIntelligence 2.2 (Cupertino, CA). Canonical simplified molecular line entry system strings (SMILES) were taken from PubChem BioAssay database (National Center for Biotechnology Information) and ChemSpider freeware (Royal Society of Chemistry, U.K.) for 205 compounds (**Table S1**). The distribution of empirical CCS values versus exact mass of the protonated molecules is shown in **Figure S1**. The variability of CCS was highlighted for isobaric species at *m/z* 280.

More than 400 molecular descriptors, obtained from Parameter Client within VCCLabs [34], were investigated (Charge descriptors, WHIM descriptors, connectivity indices, functional group counts, constitutional descriptors, eigenvalue-based indices, geometrical descriptors, and molecular properties). The number of carbon atoms (nC), average eigenvector coefficient sum from mass weighted distance matrix and van der Waals weighted distance matrix (VEm2 and VEv2), average Randic-type eigenvector-based index from distance matrix (VRD2), sum of atomic van der Waals forces (Sv), number of R=primary carbon bonds (nR=Cp), number of nine-membered rings (nR09), and the first component accessibility directional WHIM index/weighted by atomic Sanderson electronegativity (E1e) were found to be the eight most relevant descriptors in CCS prediction. These molecular descriptors were selected using the genetic algorithm (GA) feature selection to eliminate manual bias. GA-ANN has also been employed in other applications [35,36].

For the prediction optimization, the 205 compounds were partitioned randomly into training/verification/blind test sets in the ratio 68:16:16.

2.4. Network Design

All descriptors were numerical, giving eight input nodes, with "CCS" the output node. For designing the network, an exhaustive search was performed for up to 3 layers. The number of hidden units ranged from a minimum of 1 to maximum of 20 for layer 1, 13 for layer 2 and 8 for layer 3, each with a search step of 1. The "inverse test error" calculation was applied for fitness criteria, and 1200 iterations were performed for each design. The ANN program was directed to activate the best network design among the entire evaluated networks. The network design of 8–2–8–1 was selected in the end as the best.

2.5. Network Properties

The logistic function was used for hidden layer activation and output activation. In the case of input layer, there were 8 variables with 8 input nodes. The input layer simply serves as a layer that distributes inputs to the next layer "hidden layer".

2.6. Training the Network

The "Quasi-Newton" algorithm was applied for training of the network, which was continued until 500 iterations had been made. Further tests were made using the quick propagation and conjugate gradient descent training algorithms with iterations of up to 5000, but there was no advantage either in alternating the training algorithm or increasing the number of iterations from 500. The iteration speed was 103.53 iterations per second.

3. Results and discussion

3.1. Molecular descriptors

The importance of each descriptor was checked across all tests using sensitivity analysis techniques within the Alyuda NeuroIntelligence software. This was calculated as the degradation in network performance after the molecular descriptor was removed. It was found that Sv, VEm2, and VEv2 were by far the most important descriptors, accounting for more than 80% importance in the ANN performance. **Table S3** shows the importance for each descriptor, and it is worth noting that all descriptors used were of some utility in the applied ANN method. Removing even the descriptors of minimal importance (*i.e.,* VRD2 and nR09) worsened the overall prediction.

With the chosen descriptors and their associated importance for the ANN prediction being "machine-made", it is difficult to interpret their suitability in terms of chemical significance. CCS values are related to a compound's chemical structure and three-dimensional conformation, so it is interesting that internal van der Waals forces (Sv and VEv2) are two of the most important descriptors, especially as van der Waals forces have been used in previous methods for predicting ion mobilities of peptides [24].

3.2. Optimization of ANN

The number of molecular descriptors deemed necessary for ANN prediction of CCS values was determined in two steps. As the software does not allow more than 250 inputs (*i.e.,* descriptors), the ANN method was made twice in order to include all descriptors. The molecular descriptors of greatest predictive importance (>0.5% importance), calculated using sensitivity analysis techniques, were then chosen for subsequent genetic algorithm feature selection. A total of 54 descriptors were used in this step (**Table S4**) and starts using a random population of input configurations. Input configuration determines which molecular descriptors are most important and which can be ignored. In each of the following steps, it uses a process comparable to natural selection, to select superior configurations and uses them to generate a new

population. Each step successively produces a better input configuration until the optimal is found. The method is good for determining mutually required inputs and detecting interdependencies. This "machine-based" selection also eliminates any manual bias that could impact the prediction accuracy in terms of the number of descriptors as well as which are needed. In the end, following this GA selection, eight descriptors were found necessary to predict the CCS values.

Within ANN optimization, three sets were used: training, validation and blind test. The training set is the part of the input data set used for neural network training, in other words, for adjustment of network weights. The validation set is used to tune network topology or network parameters other than weights. For example, it is used to define the number of hidden units or to detect the moment when the neural network performance starts to deteriorate. The blind test set is used after the network is ready (trained), to test what errors will occur during future network application. This set is not used during training and thus can be considered as consisting of new data entered by the user for the neural network application.

The optimized architecture was found to be 8–2–8–1 using the eight molecular descriptors as inputs. This architecture was chosen on the basis of the lowest inverse mean absolute network error within the blind test set.

3.3. Performance of the CCS Predictor

The robustness of the CCS predictor and selected ANN architecture was proven by making six separate tests with compounds randomly partitioned into training, validation, and blind test sets. The individual results of each test are shown in **Supporting Information** (**Table S5–S11**), whereas the overall results are represented in **Table 1**. As can be seen, the median error value (*i.e.,* 50th percentile) was approximately 2% across all sets, with the 95th percentile being approximately 6%. Very promisingly, the errors within the blind test were low, with only 5% of the CCS errors within the set being greater than 6%. These findings prove the robustness of this method, meaning that it is not necessary to select specific compounds in each training, verification, and blind test set.

Additionally, more compounds can be added to the method without additional optimization being necessary.

Table 1. Summary of the Average Relative Error ± Standard Deviation (%) in the Prediction of CCS Values for the ANN Sets

Percentile	All sets (n=205)	Training set (n=141)	Validation set (n=32)	Blind test set (n=32)
50	1.8 ± 0.2	1.8 ± 0.2	1.5 ± 0.4	2.2 ± 0.5
60	2.3 ± 0.2	2.3 ± 0.3	1.9 ± 0.3	2.9 ± 0.4
70	2.9 ± 0.2	2.9 ± 0.3	2.5 ± 0.4	3.4 ± 0.5
80	3.6 ± 0.2	3.6 ± 0.2	3.2 ± 0.4	3.9 ± 0.6
85	4.1 ± 0.3	4.0 ± 0.2	3.8 ± 0.5	4.4 ± 0.4
90	4.7 ± 0.4	4.8 ± 0.5	4.4 ± 0.4	4.9 ± 0.5
95	5.9 ± 0.5	5.9 ± 0.7	4.9 ± 0.5	6.4 ± 1.0

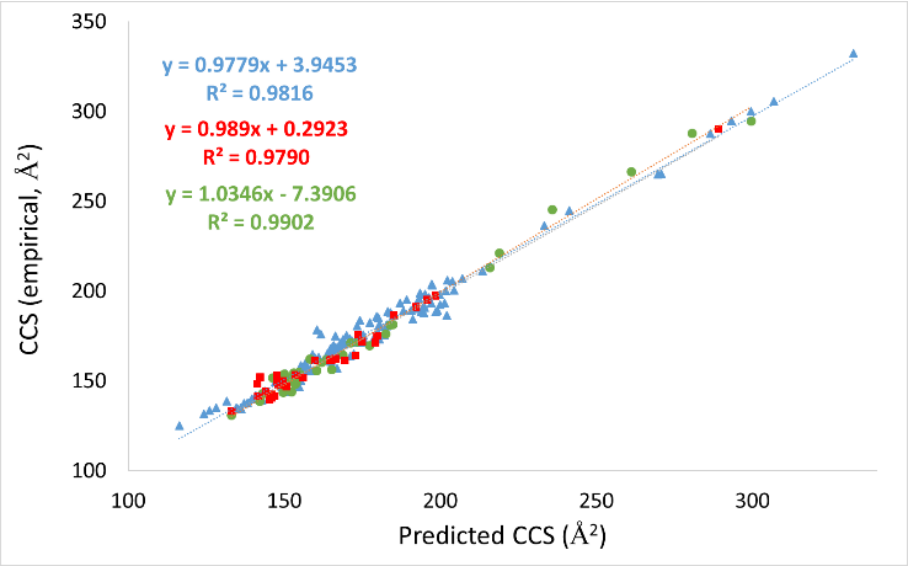


Figure 1. Correlation between measured and predicted CCS (Å²), together with linear correlations. All blue triangles are the training set, red squares are the validation set, and green circles are the blind set.

The results of one of these tests is shown in **Figure 1**, showing a great linear correlation between predicted and measured CCS values, and it must be noted that the slope is almost 1. A "t-Test: Two-Sample Assuming Unequal Variances" was performed using Microsoft Excel, and it was found that there was no statistically significant difference between the empirical and predicted CCS values with both 95% and 99% confidence (**Table S12–S13**).

Only three compounds (across all sets) had an error greater than 8% (**Figure S2**), with the greatest errors within each set being acetopromazine (−11.0%, training set), sulfapyridine (−6.9%, validation set) and chlorbromuron (5.4%, blind set).

There was no specific bias in terms of errors, with the maximum errors coming in the 140–200 $Å^2$ range merely because there were more compounds in that range.

3.4. Additional Blind Set and Adduct Prediction

A second set of 131 pesticides (none of which were used in the initial optimization of the method) was used to test the ANN as an additional means of validation. These contained both protonated molecules ($[M+H]^+$, n=64) and sodium adducts ($[M+Na]^+$, n=67), with CCS ranging between 132 and 307 $Å^2$. The prediction of CCS values of $[M+Na]^+$ was of particular importance, as due to the complex physicochemical characteristics of small molecules, some compounds may show preferential sodium adduct formation rather than protonation. It is important to note that the molecular descriptors used for the prediction were calculated from SMILES codes, which represent the neutral compound, so the impact of the sodium atom could influence the accuracy of the CCS value prediction more than protonation. **Table S14** shows the results, with 95% of the CCS values of protonated and sodiated molecules able to be predicted within an average relative error (RE) of 5.3% and 8.7%, respectively. These results demonstrate that predicted CCS values can also successfully be applied for identifying sodium adducts using the ANN predictor presented in this work, which gives extra utility when applying to real samples.

3.5. Application to Pesticide Residue Analysis

The applicability of ANNs for the prediction of CCS values was tested in blind spinach samples with the aim to check the potential confidence improvement in the identification of pesticide residues. Since no information was available (*i.e.,* spiked compounds were unknown to the analyst) a suspect and nontarget screening workflow was applied, utilizing the "Accurate Mass Screening" module within UNIFI software. Initially, an in-house database comprising more than 600 compounds (drugs of abuse, pharmaceuticals, veterinary drugs, mycotoxins, flame retardants, and pesticides) including exact mass, retention time, molecular formula, and expected fragment ions was used. Suspect compounds were tentatively identified by the software when the exact mass was within 10 ppm, retention time within 0.5 min, and at least one fragment ion was found. The exact mass and retention time threshold were initially set wide to account for matrix effect, with a later mass deviation of ≤5 ppm and retention window of ≤0.1 min needed for full confirmation, in compliance with the SANTE guidelines [37]. In addition, a nontarget workflow was applied on the basis of the exact mass only. The elemental composition of potential candidates was then searched in chemical databases, and the plausibility of the structure was thoroughly evaluated using the fragment ions obtained. Empirical CCS values were only available for 336 compounds used to build and validate the predictor. Therefore, CCS were not accessible for any other compound, but the predicted CCS values could serve as a valuable extra means of identification. Applying these workflows, 10 pesticides were tentatively identified, and the reliability in the identification was greatly improved by the use of CCS prediction (**Table 2**).

As shown in **Table 2**, four pesticides were identified after suspect-screening (*i.e.,* were included in the in-house database), whereas six additional compounds were found after nontarget screening. **Table 2** shows that deviations between the observed CCS values and the predicted values for the protonated pesticides were smaller than 6%. In addition, such deviations were also acceptable for the pesticides detected as sodium adducts.

Table 2. Pesticide Residues Tentatively Identified in the Blind Spinach Samples

	Compound	Adduct	Predicted CCS (Å²)	Observed CCS (Å²)	Deviation (%)
Suspect screening	Atrazine	$[M+H]^+$	146.29	144.75	-1.1
	Chlorantraniliprole	$[M+H]^+$	191.47	193.90	1.3
		$[M+Na]^+$		200.52	4.5
	Flonicamid	$[M+H]^+$	138.10	142.25	2.9
	Metaflumizone	$[M+H]^+$	216.58	210.05	-3.1
Non-target screening	Ametoctradin	$[M+H]^+$	171.57	173.91	1.3
		$[M+Na]^+$		188.10	8.8
	Bixafen	$[M+H]^+$	187.81	179.81	-4.4
		$[M+Na]^+$		185.70	-1.1
	Fenpropimorph	$[M+H]^+$	184.06	182.26	-1.0
	Penflufen	$[M+H]^+$	181.42	173.27	-4.7
	Pyriofenone	$[M+H]^+$	184.64	174.75	-5.3
		$[M+Na]^+$		189.36	2.5
	Valifenalate	$[M+H]^+$	194.34	196.97	1.3
		$[M+Na]^+$		198.40	2.0

The reference standard was available in our laboratory for one of the identified pesticides (atrazine), and this would obviously allow full confirmation. However, as a proof of concept, the approach developed was applied assuming that reference standards were not available in any case. This simulates future analysis where suspect and/or nontarget compounds are found, for which no experimental CCS values and/or reference standards are available.

Even when the reference standard was available, as in the case of atrazine, the CCS prediction was found to be of great value. In this sample, tentatively identified atrazine showed a mass error of 0.4 ppm and the characteristic chlorine isotopic pattern (**Figure 2**). However, we observed a deviation of 0.15 min in the retention time, which would complicate the confirmation of this compound accordingly to the SANTE guidelines [37]. The power of the CCS prediction was illustrated by the fact that the predicted CCS value (146.29 Å²) was very close (−1.1% deviation) to the observed CCS (144.75 Å²).

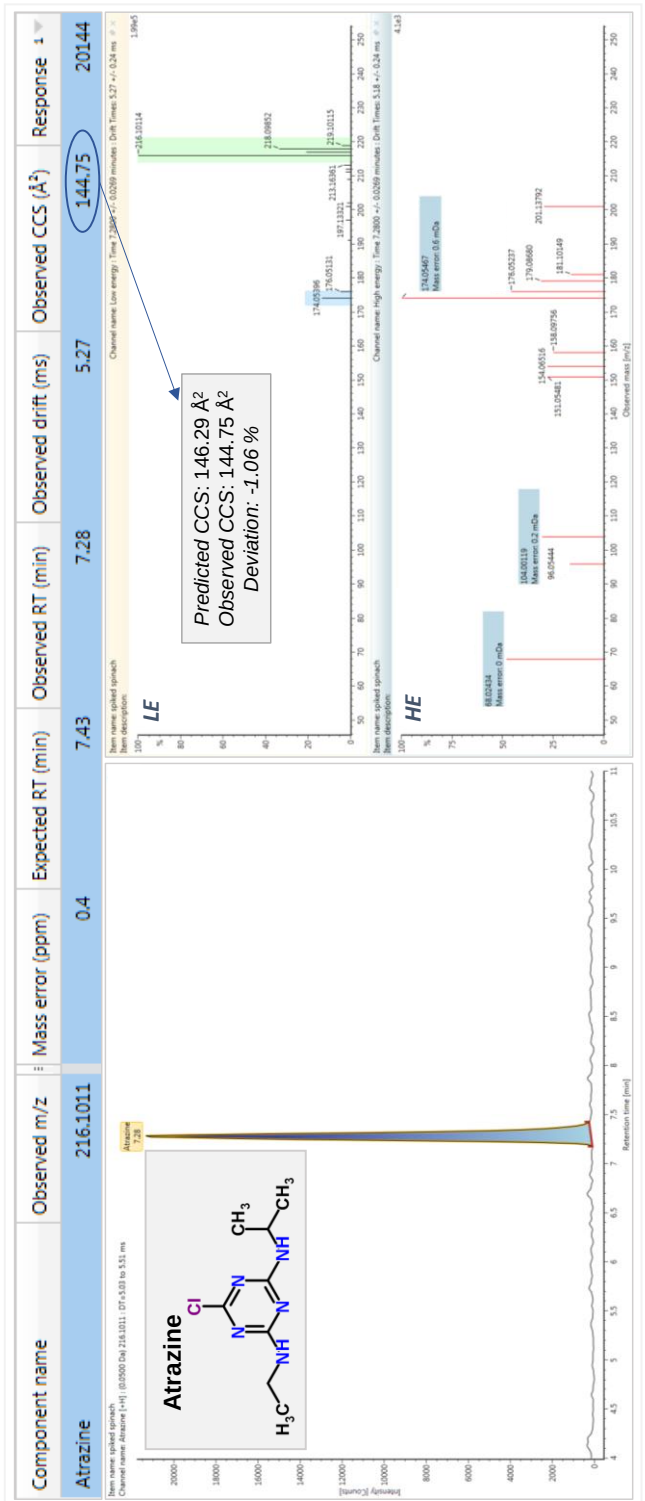


Figure 2. Tentative identification of atrazine in spinach.

After screening the spinach samples, many unidentified chromatographic peaks were observed. Because the samples were blind-prepared, the most abundant peaks and those showing halogens (*i.e.,* likely to be pesticides or other xenobiotics) were prioritized for the nontarget screening. Within UNIFI, by applying the halogen search tool in the "unknown compounds" section, candidate masses can be sorted by intensity and the number of halogens (differentiating between chlorine and bromine), resulting in 40 prioritized masses to be investigated. The online "Pesticides Common Names" database within ChemSpider was used within UNIFI to investigate possible structures. Ten masses showed only one hit, and each pesticide could subsequently be identified by matching the observed fragmentation and CCS.

One of the highest intensities belonged to a compound with an accurate *m/z* of 421.1491, and an observed CCS of 198.40 $Å^2$ (**Figure 3**). 421.1491 corresponded to a $[M+Na]^+$ adduct, with the $[M+H]^+$ and $[M+K]^+$ also observed. When elucidating possible structures, the most intense peak $[M+Na]^+$ was used, and the elemental composition was found to be $C_{19}H_{27}ClN_2O_5Na$. This formula resulted in a single hit corresponding to valifenalate as possible compound, which also matches with the observed fragmentation. However, as no reference standard was available, predicted CCS was used as an additional parameter to obtain more confidence in its identity. The predicted CCS value of 194.34 $Å^2$ for valifenalate had an error within 2.0% from the observed CCS value. This was well within the relative errors of 6% and 9% calculated for 95% of the CCS values of the protonated and sodium adducts, respectively.

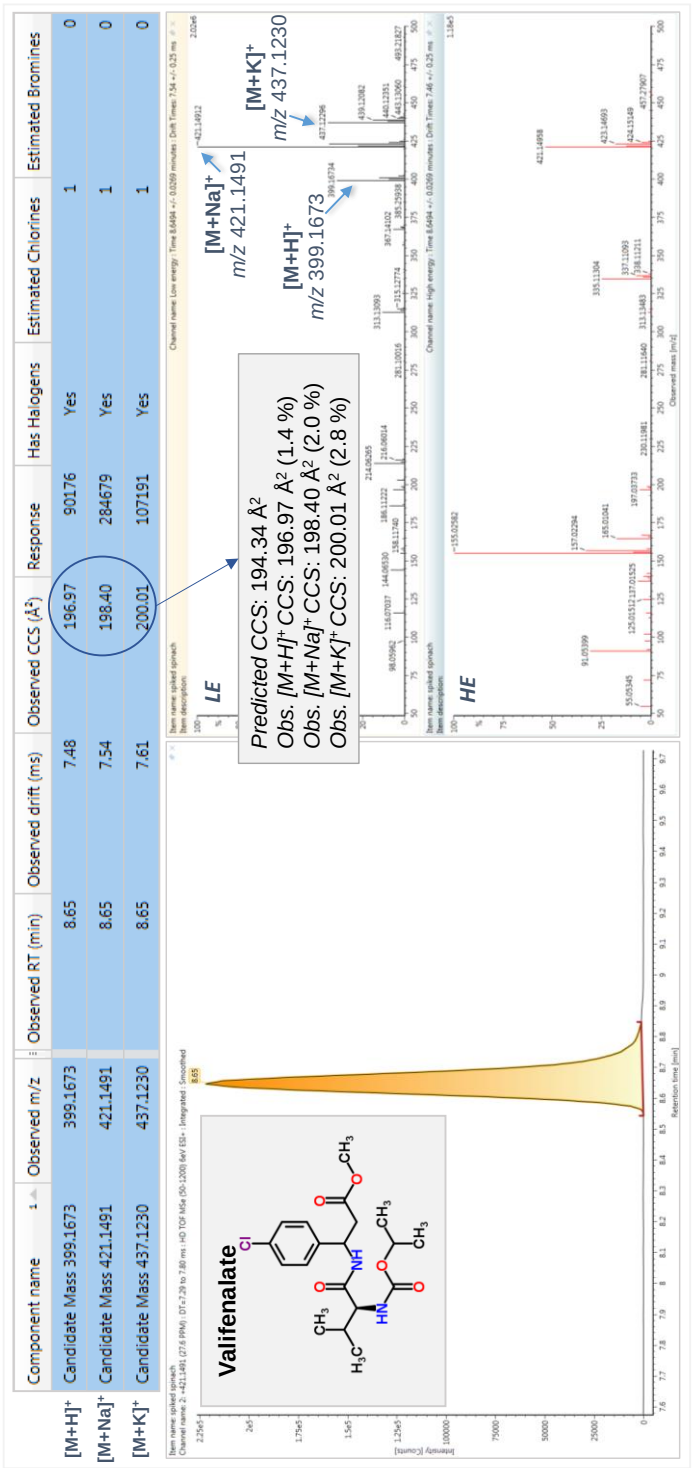


Figure 3. Tentative identification of valifenalate.

The detection of three adducts within the same drift time and retention time window in **Figure 3** was an interesting finding. With sodium and potassium ions being larger than a proton, it is expected that the CCS value would be higher and even fall outside the drift time window selected for this analysis (±0.25 ms). However, it appears that valifenalate has a cavity within which a proton, sodium, and potassium can fit without changing the overall shape (and thus drift time and CCS) markedly.

In general, a drift time window of ±0.25 ms means a higher selectivity as compounds, not corresponding to the peak of interest, are filtered out. However, important information regarding related adducts can also be ignored. In order to determine whether the drift time alignment can affect the detection of sodium adducts, components in the nontarget approach of the spinach samples were more closely investigated, with 22 sodium/proton pairs found. It was observed that there was a difference in drift time of up to 3.4 ms between the protonated and sodium adducts, with CCS differences of around 5%, with only three beyond 8%. **Figure 4** illustrates the tentative identification of pyriofenone. The CCS value of pyriofenone was predicted and fell within the 6% error for the protonated molecule. However, the sodium adduct of this pesticide has a difference in drift time with the protonated molecule of 0.58 ms, and therefore, this adduct was not seen in the drift time-aligned spectrum (**Figure 4A**).

Yet, when drift time alignment was not applied (**Figure 4B**) the sodium adduct and its CCS value (189.36 $Å^2$) could also be observed with a deviation of 2.5%. This, again, demonstrates the applicability of CCS prediction for nontarget analysis, even when some adducts are missing due to drift time alignment.

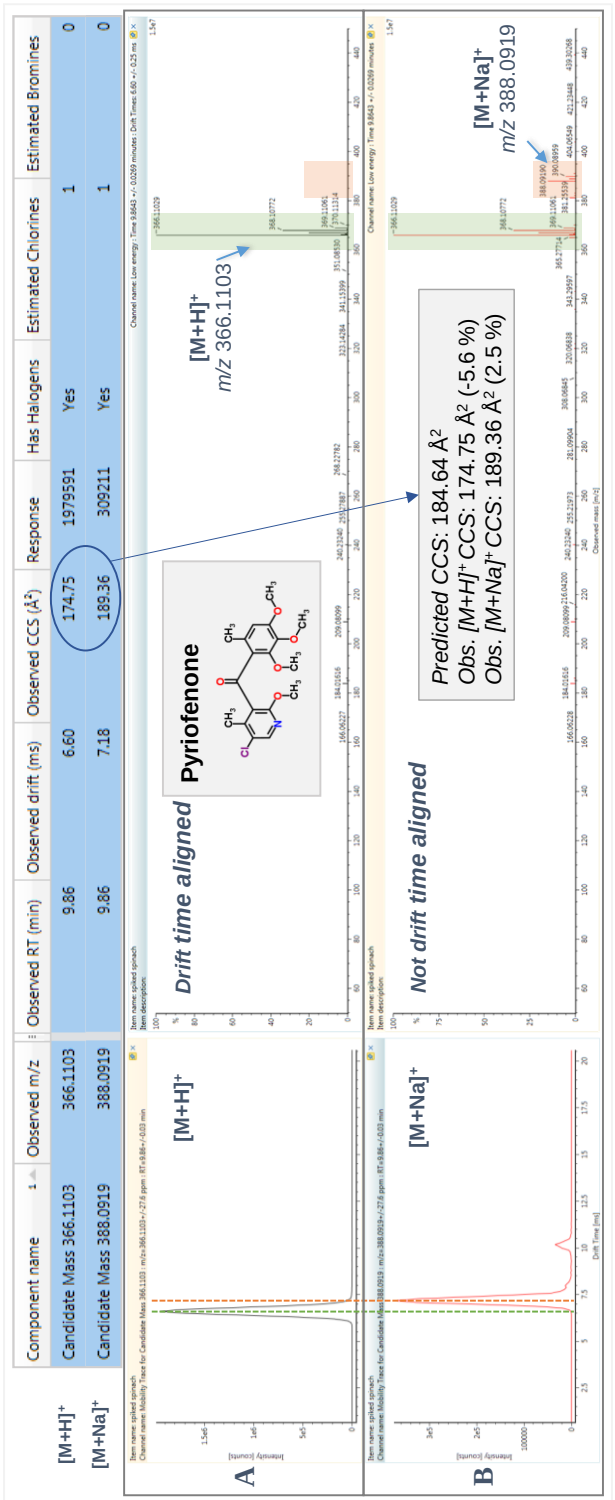


Figure 4. Driftograms of the protonated molecule of pyriofenone (A) and sodium adduct (B), together with associated mass spectra and CCS values.

In summary, from the 10 pesticides tentatively identified in the blind spinach samples, nine were subsequently confirmed as they were used for spiking the samples (**Table S2**), while one compound (dinotefuran) was not detected. This could possibly be explained by biological and/or chemical degradation during storage [38]. Furthermore, fenpropimorph was apparently present in the "blank" spinach used in this study.

In this study, a common pesticide database was used for searching logical candidates. However, when a wider and generic database is applied, the number of potential candidates per mass increases. In this case, CCS filtering used prior to fragment ion information would allow to discard around 50% of the "positives" found, which is in agreement with the accuracy (6%; 95th percentile) of the CCS predictor. **Figure S1**, for example, show that the average CCS at *m/z* 280 was 160 Å^2 with a 35 Å^2 range. The prediction accuracy resulted in a 19.2 Å^2 window (±6% of 160 Å2), representing around half of the range. Nevertheless, one of the aims of this work was to use CCS prediction in order to gain confidence in the identification of pesticides in food analysis. Therefore, the applied strategy combines the resolving powers offered by LC, IMS, and HRMS in order to discriminate as many false positives as possible in spinach samples and allow the identification of detected pesticides with high reliability.

4. Conclusions

This work has presented the development and utility of CCS prediction in the identification of pesticide residues in food. CCS values could be predicted for protonated molecules and successfully be applied for identifying sodium adducts with high accuracy. It is important to note that this approach is completely universal for small molecules. CCS values are unaffected by the sample matrix, and their accuracy encourages future application in wide-scope screening of any sample matrix, including for compounds for which standards are not commonly available. This approach could be used to investigate not only parent compounds but also possible metabolites. Hence, there is a great potential in the ANN-based CCS prediction for large screening of organic contaminants in applied fields, such as environmental pollution and food-safety. This tool, in combination with the intrinsic properties of high-resolution tandem mass spectrometry (accurate-mass full spectrum acquisition and relevant information on product ion) notably facilitates the identification process and improves its reliability in target, suspect, and nontarget analysis.

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Supplementary data

In this section, 14 tables and two figures are included to have supportive information on the written text. Table S1 shows the complete list of chemicals used in this study. Table S2 indicates the list of chemicals spiked in the samples for analysis. Table S3 shows the importance of each descriptor in the prediction model developed. Table S4 indicates the list of 54 molecular descriptors chosen for genetic-algorithm feature selection. Tables S5-S11 show the different prediction accuracies depending on the percentile of choice. Tables S12-S13 show the statistical significance of the correlation between empirical and predicted CCS. Table S14 show the errors obtained for the blind set of pesticides used for validation. Figure S1 plots the empirical CCS value *vs.* the exact mass of the molecules used for model development. Figure S2 show the relative errors for CCS values of all compounds.

Supplementary data to this article can be found, in the online version, at https://doi.org/10.1021.acs.analchem.7b00741 and in this chapter after the section “References”.

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Supplementary data

Table S1. Compound names, empirical CCS values and SMILES of the 205 protonated molecules used to develop the ANN predictor.

#	Compound name	Exact mass (*m/z*)	Emperical CCS (Å²)	SMILES
1	1,4-androstadiene-3,17-dione (ADD)	285.1849	166.50	O=C\1\C=C/[C@]3(/C(=C/1)CC[C@H]4[C@@H]2CCC(=O)[C@]2(CC[C@H]34)C)C
2	16β-hydroxystanozolol	345.2537	192.10	C[C@]12CC[C@H]3[C@H]([C@@H]1C[C@@H]([C@]2(C)O)O)CC[C@@H]4[C@@]3(Cc5cn[nH]c5C4)C
3	17α-Methyltestosterone	303.2319	173.70	C[C@]12CCC(=O)C=C1CC[C@@H]3[C@@H]2CC[C@]4([C@H]3CC[C@]4(C)O)C
4	17α-Nortestosterone	275.2006	165.70	C[C@]12CC[C@H]3[C@H]([C@@H]1CC[C@H]2O)CCC4=CC(=O)CC[C@@H]34
5	17α-Trenbolone	271.1693	163.30	CC12C=CC3=C4CCC(=O)C=C4CCC3C1CCC2O
6	17β-Nortestosterone	275.2006	165.70	C[C@]12CC[C@H]3[C@H]([C@@H]1CC[C@H]2O)CCC4=CC(=O)CC[C@@H]34
7	17β-Trenbolone	271.1693	163.30	C[C@]12C=CC3=C4CCC(=O)C=C4CC[C@H]3[C@@H]1CC[C@@H]2O
8	Acetamiprid	223.0745	150.24	C/C(=N\C#N)/N(C)CC1=CN=C(C=C1)Cl
9	Acetopromazine	327.1526	160.50	CC(=O)c1ccc2c(c1)N(c3ccccc3S2)CCCN(C)C
10	Aldicarb sulfoxide	207.0798	145.40	CC(C)(C=NOC(=O)NC)S(=O)C
11	Azaperone	328.1820	180.30	C1CN(CCN1CCCC(=O)C2=CC=C(C=C2)F)C3=CC=CC=N3
12	Azoxystrobin	404.1241	195.75	CO/C=C(\C1=CC=CC=C1OC2=NC=NC(=C2)OC3=CC=CC=C3C#N)/C(=O)OC
13	Beclomethasone	409.1776	189.20	C[C@H]1C[C@H]2[C@@H]3CCC4=CC(=O)C=C[C@@]4([C@]3([C@H](C[C@@]2([C@]1(C(=O)CO)O)C)O)Cl)C
14	Benthiavalicarb	340.1126	173.38	CC(C)C(C(=O)NC(C)c1nc2ccc(cc2s1)F)NC(=O)O
15	Boscalid	343.0399	171.67	C1=CC=C(C(=C1)C2=CC=C(C=C2)Cl)NC(=O)C3=C(N=CC=C3)Cl
16	Bromchlorbuterol	321.0364	165.20	CC(C)(C)NCC(C1=CC(=C(C(=C1)Br)N)Cl)O
17	Brombuterol	364.9859	167.00	CC(C)(C)NCC(c1cc(c(c(c1)Br)N)Br)O
18	Buprofezin	306.1635	173.58	CC(C)N1/C(=N/C(C)(C)C)/SCN(C1=O)C2=CC=CC=C2
19	Butafenacil	475.0878	208.59	CC(C)(C(=O)OCC=C)OC(=O)C1=C(C=CC(=C1)N2C(=O)C=C(N(C2=O)C)C(F)(F)F)Cl
20	Carazolol	299.1754	165.50	CC(C)NCC(COC1=CC=CC2=C1C3=CC=CC=C3N2)O
21	Carbendazim	192.0768	134.94	COC(=O)NC1=NC2=CC=CC=C2N1
22	Carboxin	236.0740	149.35	CC1=C(SCCO1)C(=O)NC2=CC=CC=C2
23	Carprofen	274.0629	156.10	CC(c1ccc2c3cc(ccc3[nH]c2c1)Cl)C(=O)O
24	Chlorbromuron	292.9687	152.25	CN(C(=O)NC1=CC(=C(C=C1)Br)Cl)OC
25	Chloridazon (Pyrazon)	222.0429	141.59	C1=CC=C(C=C1)N2C(=O)C(=C(C=N2)N)Cl
26	Chloroxuron	291.0895	172.24	CN(C)C(=O)Nc1ccc(cc1)Oc2ccc(cc2)Cl

#	Compound name	Exact mass (m/z)	Emperical CCS (Å²)	SMILES
27	Chlorpromazine	319.1030	169.10	CN(C)CCCN1C2=CC=CC=C2SC3=C1C=C(C=C3)Cl
28	Chlorsulfuron	358.0370	175.29	CC1=NC(=NC(=N1)OC)NC(=O)NS(=O)(=O)C2=CC=CC=C2Cl
29	Chlortetracycline	479.1216	203.90	C[C@]1(c2c(ccc(c2C(=O)C3=C([C@]4([C@@H](C[C@@H]31)[C@@H](C(=C(C4=O)C(=N)O)O)N(C)C)O)O)O)Cl)O
30	Chlortoluron	213.0789	145.96	Cc1ccc(cc1Cl)/N=C(\N(C)C)/O
31	Cimaterol	220.1444	155.40	CC(C)NCC(c1ccc(c(c1)C#N)N)O
32	Clenbuterol	277.0869	159.20	Clc1cc(cc(Cl)c1N)C(O)CNC(C)(C)C
33	Clencyclohexerol	319.0975	164.70	c1c(cc(c(c1Cl)N)Cl)C(CNC2CCC(CC2)O)O
34	Clenpenterol	291.1025	165.00	CCC(C)(C)NCC(O)c1cc(Cl)c(N)c(Cl)c1
35	Clobetasol	411.1733	201.29	C[C@H]1C[C@H]2[C@@H]3CCC4=CC(=O)C=C[C@@]4([C@]3([C@H](C[C@@]2([C@]1(C(=O)CCl)O)C)O)F)C
36	Clofentezine	303.0199	161.90	c1ccc(c(c1)c2nnc(nn2)c3ccccc3Cl)Cl
37	Clostebol	323.1772	173.60	C[C@]12CC[C@H]3[C@H]([C@@H]1CC[C@@H]2O)CCC4=C(C(=O)CC[C@]34C)Cl
38	Cortisone	361.2010	184.00	C[C@]12CCC(=O)C=C1CC[C@@H]3[C@@H]2C(=O)C[C@]4([C@H]3CC[C@@]4(C(=O)CO)O)C
39	Cyazofamid	325.0521	166.69	Cc1ccc(cc1)c2c(nc(n2S(=O)(=O)N(C)C)C#N)Cl
40	Cyprodinil	226.1339	152.07	CC1=CC(=NC(=N1)NC2=CC=CC=C2)C3CC3
41	Cyromazine	167.1040	133.23	C1CC1NC2=NC(=NC(=N2)N)N
42	Dapsone	249.0692	149.90	C1=CC(=CC=C1N)S(=O)(=O)C2=CC=C(C=C2)N
43	DEET	192.1383	142.48	CCN(CC)C(=O)c1cccc(c1)C
44	Demeton-S-methyl-sulfone	263.0171	143.66	CCS(=O)(=O)CCSP(=O)(OC)OC
45	Diafenthiuron	385.2308	199.75	CC(C)C1=CC(=CC(=C1NC(=S)NC(C)(C)C)C(C)C)OC2=CC=CC=C2
46	Dichlorvos	220.9532	133.07	COP(=O)(OC)OC=C(Cl)Cl
47	Diclofenac	296.0240	157.20	c1ccc(c(c1)CC(=O)O)Nc2c(cccc2Cl)Cl
48	Difenoconazole	406.0720	198.48	CC1COC(O1)(Cn2cncn2)c3ccc(cc3Cl)Oc4ccc(cc4)Cl
49	Difloxacine	400.1497	192.30	Fc1ccc(cc1)N\3c2cc(c(F)cc2C(=O)C(/C(=O)O)=C/3)N4CCN(C)CC4
50	Diflubenzuron	311.0393	160.98	C1=CC(=C(C(=C1)F)C(=O)NC(=O)NC2=CC=C(C=C2)Cl)F
51	Dimethomorph I	388.1310	199.03	COc1ccc(cc1OC)/C(=C/C(=O)N2CCOCC2)/c3ccc(cc3)Cl
52	Dimetridazole	142.0611	116.40	CC1=NC=C(N1C)[N+](=O)[O-]
53	Doxycycline	445.1605	197.40	C[C@@H]1[C@H]2[C@@H]([C@H]3[C@@H](C(=C(C(=O)[C@]3(C(=C2C(=O)C4=C1C=CC=C4O)O)O)C(=O)N)O)N(C)C)O

#	Compound name	Exact mass (*m/z*)	Emperical CCS (Å²)	SMILES
54	Dulcoside A	789.3903	270.75	C[C@H]1[C@@H]([C@H]([C@H]([C@@H](O1)O[C@@H]2[C@H]([C@@H]([C@H](O[C@H]2O[C@@]34CC[C@H]5[C@@]6(CCC[C@@]([C@H]6CC[C@]5(C3)CC4=C)(C)C(=O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)CO)O)O)O)C)CO)O)O)O)O)O
55	Enrofloxacine	360.1718	194.80	O=C(O)\C3=C\N(c2cc(N1CCN(CC)CC1)c(F)cc2C3=O)C4CC4
56	Ethirimol	210.1061	150.65	CCCCC1=C(N=C(NC1=O)NCC)C
57	Ethoprophos	243.0637	149.27	CCCSP(=O)(OCC)SCCC
58	Fenamidone	312.1165	171.15	C[C@@]1(C(=O)N(C(=N1)SC)NC2=CC=CC=C2)C3=CC=CC=C3
59	Fenamiphos sulfone	336.1029	179.48	CCOP(=O)(NC(C)C)Oc1ccc(c(c1)C)S(=O)(=O)C
60	Fenbuconazole	337.1215	182.44	C1=CC=C(C=C1)C(CCC2=CC=C(C=C2)Cl)(CN3C=NC=N3)C#N
61	Fenhexamid	302.0709	164.82	CC1(CCCCC1)C(=O)NC2=C(C(=C(C=C2)O)Cl)Cl
62	Fenoterol	304.1543	180.40	CC(Cc1ccc(cc1)O)NCC(c2cc(cc(c2)O)O)O
63	Fenoxycarb	302.1387	178.44	CCOC(=O)NCCOC1=CC=C(C=C1)OC2=CC=CC=C2
64	Fenpropidin	274.2529	166.39	CC(CC1=CC=C(C=C1)C(C)(C)C)CN2CCCCC2
65	Fenpyroximate	422.2074	215.89	CC1=NN(C(=C1/C=N/OCC2=CC=C(C=C2)C(=O)OC(C)(C)C)OC3=CC=CC=C3)C
66	Fipronil	436.9460	180.77	C1=C(C=C(C(=C1Cl)N2C(=C(C(=N2)C#N)S(=O)C(F)(F)F)N)Cl)C(F)(F)F
67	Firocoxib	337.1104	182.10	CC1(C(=C(C(=O)O1)OCC2CC2)c3ccc(cc3)S(=O)(=O)C)C
68	Fluazafop-P-butyl	384.1417	194.80	CCCCOC(=O)[C@@H](C)Oc1ccc(cc1)Oc2ccc(cn2)C(F)(F)F
69	Fluazifop	328.0791	173.47	CC(C(=O)O)Oc1ccc(cc1)Oc2ccc(cn2)C(F)(F)F
70	Flufenoxuron	489.0435	204.46	C1=CC(=C(C(=C1)F)C(=O)NC(=O)NC2=C(C=C(C=C2)OC3=C(C=C(C=C3)C(F)(F)F)Cl)F)F
71	Flumequine	262.0874	147.60	CC1CCC2=C3N1C=C(C(=O)C3=CC(=C2)F)C(=O)O
72	Flumethasone	411.1978	193.20	C[C@@H]1C[C@H]2[C@@H]3C[C@@H](C4=CC(=O)C=C[C@@]4([C@]3([C@H](C[C@@]2([C@]1(C(=O)CO)O)C)O)F)C)F
73	Flunixin	297.0845	159.10	CC1=C(C=CC=C1NC2=C(C=CC=N2)C(=O)O)C(F)(F)F
74	Fluroxypyr	254.9734	140.69	C(C(=O)O)OC1=NC(=C(C(=C1Cl)N)Cl)F
75	Flutolanil	324.1206	175.45	CC(C)OC1=CC=CC(=C1)NC(=O)C2=CC=CC=C2C(F)(F)F
76	Flutriafol	302.1099	163.97	C1=CC=C(C(=C1)C(CN2C=NC=N2)(C3=CC=C(C=C3)F)O)F
77	Formetanate	222.1237	153.76	CNC(=O)OC1=CC=CC(=C1)N=CN(C)C
78	Furalaxyl	302.1387	167.15	CC1=C(C(=CC=C1)C)N(C(C)C(=O)OC)C(=O)C2=CC=CO2
79	Furathiocarb	383.1635	190.73	CCCCOC(=O)N(C)SN(C)C(=O)OC1=CC=CC2=C1OC(C2)(C)C

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80	Gamithromycin	777.5471	280.60	CCCN1C[C@@H]([C@H]([C@]([C@H](OC(=O)[C@@H]([C@H]([C@@H]([C@H]([C@](C[C@H]1C)(C)O)O[C@H]2[C@@H]([C@H](C[C@H](O2)C)N(C)C)O)C)O[C@H]3C[C@@]([C@H]([C@@H](O3)C)O)(C)OC)C)CC)(C)O)O)C
81	Haloperidol	376.1474	193.90	c1cc(ccc1C(=O)CCCN2CCC(CC2)(c3ccc(cc3)Cl)O)F
82	Haloxyfop	362.0401	179.86	C2(OC1=CC=C(OC(C)C(=O)O)C=C1)=C(Cl)C=C(C(F)(F)F)C=N2
83	Haloxyfop-2-ethoxyethyl	434.0977	193.53	CCOCCOC(=O)C(C)OC1=CC=C(C=C1)OC2=C(C=C(C=N2)C(F)(F)F)Cl
84	Heptenophos	251.0234	144.04	COP(=O)(OC)OC1=C(C2C1CC=C2)Cl
85	Hexaconazole	314.0821	171.26	CCCCC(CN1C=NC=N1)(C2=C(C=C(C=C2)Cl)Cl)O
86	Hexaflumuron	460.9889	188.22	C1=CC(=C(C(=C1)F)C(=O)NC(=O)NC2=CC(=C(C(=C2)Cl)OC(C(F)F)(F)F)Cl)F
87	Hexythiazox	353.1085	183.83	Clc1ccc(cc1)C3SC(=O)N(C(=O)NC2CCCCC2)C3C
88	Hydroxymetronidazole	188.0666	128.20	c1c(n(c(n1)CO)CCO)[N+](=O)[O-]
89	Hydroxythiabendazole	218.0383	137.20	C1=CC2=C(C=C1O)NC(=N2)C3=CSC=N3
90	Imazalil	297.0556	164.99	C=CCOC(CN1C=CN=C1)C2=C(C=C(C=C2)Cl)Cl
91	Imidacloprid	256.0596	149.62	C1CN(/C(=N/[N+](=O)[O-])/N1)CC2=CN=C(C=C2)Cl
92	Indoprofen	282.1125	166.40	CC(c1ccc(cc1)N2Cc3ccccc3C2=O)C(=O)O
93	Indoxacarb	528.0780	207.03	COC(=O)[C@]12Cc3cc(ccc3C1=NN(CO2)C(=O)N(c4ccc(cc4)OC(F)(F)F)C(=O)OC)Cl
94	Ipronidazole	170.0924	126.10	CC(C)c1ncc(n1C)[N+](=O)[O-]
95	Isoproturon	207.1492	150.75	CC(C)C1=CC=C(C=C1)NC(=O)N(C)C
96	Isoxaben	333.1809	184.93	CCC(C)(CC)C1=NOC(=C1)NC(=O)C2=C(C=CC=C2OC)OC
97	Josamycin	828.4740	306.80	C[C@@H]1C/C=C/C=C/[C@@H]([C@@H](C[C@@H]([C@@H]([C@H]([C@@H](CC(=O)O1)OC(=O)C)OC)O[C@H]2[C@@H]([C@H]([C@@H]([C@H](O2)C)O[C@H]3C[C@@]([C@H]([C@@H](O3)C)OC(=O)CC(C)C)(C)O)N(C)C)O)CC=O)C)O
98	Ketoprofen	255.1016	155.40	CC(C1=CC(=CC=C1)C(=O)C2=CC=CC=C2)C(=O)O
99	Levamisole	205.0794	138.20	C1CSC2=N[C@H](CN21)C3=CC=CC=C3
100	Lincomycin	407.2210	201.80	CCC[C@@H]1C[C@H](N(C1)C)C(=O)N[C@@H]([C@@H]2[C@@H]([C@@H]([C@H]([C@H](O2)SC)O)O)O)[C@@H](C)O
101	Linuron	249.0192	149.63	CN(C(=O)NC1=CC(=C(C=C1)Cl)Cl)OC
102	Lufenuron	510.9857	198.53	C1=CC(=C(C(=C1)F)C(=O)NC(=O)NC2=CC(=C(C=C2Cl)OC(C(C(F)(F)F)F)(F)F)Cl)F
103	Mabuterol	311.1133	168.40	CC(C)(C)NCC(C1=CC(=C(C(=C1)Cl)N)C(F)(F)F)O
104	Mandipropamid	412.1310	195.15	COC1=C(C=CC(=C1)CCNC(=O)C(C2=CC=C(C=C2)Cl)OCC#C)OCC#C

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105	Marbofloxacin	363.1463	179.60	CN1CCN(CC1)C2=C(C=C3C4=C2OCN(N4C=C(C3=O)C(=O)O)C)F
106	Mefenamic acid	242.1176	150.50	CC1=C(C(=CC=C1)NC2=CC=CC=C2C(=O)O)C
107	Meloxicam	352.0420	174.90	Cc1cnc(s1)/N=C(/C2=C(c3ccccc3S(=O)(=O)N2C)O)\O
108	Mepanipyrim	224.1182	152.07	CC#CC1=NC(=NC(=C1)C)NC2=CC=CC=C2
109	Mesotrione	340.0485	175.66	CS(=O)(=O)C1=CC(=C(C=C1)C(=O)C2C(=O)CCCC2=O)[N+](=O)[O-]
110	Metalaxyl	280.1543	159.96	CC1=C(C(=CC=C1)C)N(C(C)C(=O)OC)C(=O)COC
111	Metamitron	203.0927	140.93	CC1=NN=C(C(=O)N1N)C2=CC=CC=C2
112	Metconazole	320.1524	168.62	CC1(CCC(C1(CN2C=NC=N2)O)CC3=CC=C(C=C3)Cl)C
113	Methabenzthiazuron	222.0696	142.47	CNC(=O)N(C)c1nc2ccccc2s1
114	Methylboldenone	301.2162	177.30	C[C@]12CC[C@H]3[C@H]([C@@H]1CC[C@@H]2OC)CCC4=CC(=O)C=C[C@]34C
115	Metobromuron	259.0077	145.41	CN(C(=O)NC1=CC=C(C=C1)Br)OC
116	Metolachlor	284.1412	158.23	CCC1=CC=CC(=C1N(C(C)COC)C(=O)CCl)C
117	Metribuzin	215.0961	146.95	CC(C)(C)C1=NN=C(N(C1=O)N)SC
118	Metronidazole	172.0717	124.20	Cc1ncc(n1CCO)[N+](=O)[O-]
119	Monolinuron	215.0582	142.90	CN(C(=O)NC1=CC=C(C=C1)Cl)OC
120	Morantel	221.1107	149.70	Cc1ccsc1/C=C/C2=NCCCN2C
121	Nalidixic acid	233.0921	141.40	CCN1C=C(C(=O)C2=C1N=C(C=C2)C)C(=O)O
122	Naproxen	231.1016	147.10	C[C@@H](c1ccc2cc(ccc2c1)OC)C(=O)O
123	Niflumic acid	283.0689	156.80	c1cc(cc(c1)Nc2c(cccn2)C(=O)O)C(F)(F)F
124	Nitenpyram	271.0956	160.32	CCN(CC1=CN=C(C=C1)Cl)/C(=C/[N+](=O)[O-])/NC
125	Norfloxacine	320.1405	171.00	O=C(O)\C2=C\N(c1cc(c(F)cc1C2=O)N3CCNCC3)CC
126	Novaluron	493.0196	196.35	C1=CC(=C(C(=C1)F)C(=O)NC(=O)NC2=CC(=C(C=C2)OC(C(OC(F)(F)F)F)(F)F)Cl)F
127	Omethoate	214.0297	134.62	C/N=C(\CSP(=O)(OC)OC)/O
128	Oxadiazon	345.0767	182.17	CC(C)OC1=C(C=C(C(=C1)N2C(=O)OC(=N2)C(C)(C)C)Cl)Cl
129	Oxolinic acid	262.0710	147.80	CCN1C=C(C(=O)C2=CC3=C(C=C21)OCO3)C(=O)O
130	Oxytetracycline	461.1555	202.30	C[C@@]1([C@H]2[C@@H]([C@H]3[C@@H](C(=C(C(=O)[C@]3(C(=C2C(=O)C4=C1C=CC=C4O)O)O)C(=O)N)O)N(C)C)O)O
131	Paclobutrazol	294.1368	168.73	CC(C)(C)[C@@H]([C@H](CC1=CC=C(C=C1)Cl)N2C=NC=N2)O
132	Paraoxon-ethyl	276.0632	156.76	CCOP(=O)(OCC)Oc1ccc(cc1)[N+](=O)[O-]

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133	Pencycuron	329.1415	179.71	Clc1ccc(cc1)CN(C(=O)Nc2ccccc2)C3CCCC3
134	Picoxystrobin	368.1104	177.50	CO/C=C(\C1=CC=CC=C1COC2=CC=CC(=N2)C(F)(F)F)/C(=O)OC
135	Pirimicarb	239.1503	154.68	CC1=C(N=C(N=C1OC(=O)N(C)C)N(C)C)C
136	Piroxicam	332.0700	170.90	CN1C(=C(c2ccccc2S1(=O)=O)O)/C(=N/c3ccccn3)/O
137	Prednisone	359.1853	180.10	C[C@]12CC(=O)[C@H]3[C@H]([C@@H]1CC[C@@]2(C(=O)CO)O)CCC4=CC(=O)C=C[C@]34C
138	Procaterol	291.1703	168.00	CC(C)NC(CC)C(O)c2ccc(O)c1NC(=O)C=Cc12
139	Prochloraz	376.0381	161.68	O=C(N(CCOc1c(Cl)cc(Cl)cc1Cl)C(C)C)n2ccnc2
140	Propachlor	212.0837	142.07	CC(C)N(C1=CC=CC=C1)C(=O)CCl
141	Propionylpromazine	341.1682	182.80	CCC(=O)c1ccc2c(c1)N(c3ccccc3S2)CCCN(C)C
142	Propyphenazone	231.1492	150.00	O=C2\C(=C(/N(N2c1ccccc1)C)C)C(C)C
143	Proquinazid	373.0407	169.35	Ic2ccc1\N=C(\OCCC)N(C(=O)c1c2)CCC
144	Prosulfocarb	252.1417	156.95	CCCN(CCC)C(=O)SCC1=CC=CC=C1
145	Prosulfuron	420.0948	187.01	Cc1nc(nc(n1)OC)NC(=O)NS(=O)(=O)c2ccccc2CCC(F)(F)F
146	Pymetrozine	218.1036	150.71	CC1=NNC(=O)N(C1)/N=C/C2=CN=CC=C2
147	Pyraclostrobin	388.1059	183.19	COC(=O)N(c1ccccc1COc2ccn(n2)c3ccc(cc3)Cl)OC
148	Pyrantel	207.0950	143.00	CN1CCCN=C1/C=C/c2cccs2
149	Pyridate	379.1242	199.90	CCCCCCCCSC(=O)OC1=CC(=NN=C1C2=CC=CC=C2)Cl
150	Pyrimethanil	200.1182	142.95	CC1=CC(=NC(=N1)NC2=CC=CC=C2)C
151	Pyriproxifen	322.1438	182.76	CC(COc1ccc(cc1)Oc2ccccc2)Oc3ccccn3
152	Quinalphos	299.0614	165.10	CCOP(=S)(OCC)OC1=NC2=CC=CC=C2N=C1
153	Quinclorac	241.9770	139.54	c1cc(c(c2c1cc(cn2)Cl)C(=O)O)Cl
154	Ractopamine	302.1751	168.90	CC(CCC1=CC=C(C=C1)O)NCC(C2=CC=C(C=C2)O)O
155	Rebaudioside A	967.4381	299.48	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)CO)O)O)O)(C)C(=O)O[C@H]8[C@@H]([C@H]([C@@H]([C@H](O8)CO)O)O)O
156	Rebaudioside B	805.3852	261.19	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)CO)O)O)O)(C)C(=O)O

#	Compound name	Exact mass (*m/z*)	Emperical CCS (Å²)	SMILES
157	Rebaudioside C	951.4432	299.49	C[C@H]1[C@@H]([C@H]([C@H]([C@@H](O1)O[C@@H]2[C@H]([C@@H]([C@H](O[C@H]2O[C@@]34CC[C@H]5[C@@]6(CCC[C@@]([C@H]6CC[C@]5(C3)CC4=C)(C)C(=O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)CO)O)O)O)C)CO)O)O[C@H]8[C@@H]([C@H]([C@@H]([C@H](O8)CO)O)O)O)O)O)O
158	Rebaudioside E	967.4381	289.20	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)(C)C(=O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)CO)O)O)O[C@H]8[C@@H]([C@H]([C@@H]([C@H](O8)CO)O)O)O)O
159	Rebaudioside F	937.4275	293.18	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)O[C@H]7[C@@H]([C@H]([C@@H](CO7)O)O)O)(C)C(=O)O[C@H]8[C@@H]([C@H]([C@@H]([C@H](O8)CO)O)O)O
160	Ritodrine	288.1594	165.80	C[C@@H]([C@@H](c1ccc(cc1)O)O)NCCc2ccc(cc2)O
161	Ronidazole	201.0618	131.60	CN1C(=CN=C1COC(=O)N)[N+](=O)[O-]
162	Rotenone	395.1489	195.01	CC(=C)C1Cc2c(ccc3c2OC4COc5cc(c(cc5C4C3=O)OC)OC)O1
163	Rubusoside	643.3324	241.31	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O)O)(C)C(=O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)O
164	Salbutamol	240.1594	158.30	CC(C)(C)NCC(C1=CC(=C(C=C1)O)CO)O
165	Sarafloxacine	386.1311	202.00	Fc1ccc(cc1)N\3c2cc(c(F)cc2C(=O)C(/C(=O)O)=C/3)N4CCNCC4
166	Simazine	202.0854	142.09	CCNC1=NC(=NC(=N1)Cl)NCC
167	Spirotetramat	374.1962	189.99	CCOC(=O)OC1=C(C(=O)NC12CCC(CC2)OC)C3=C(C=CC(=C3)C)C
168	Stanozolol	329.2587	185.20	C[C@]12CC[C@H]3[C@H]([C@@H]1CC[C@]2(C)O)CC[C@@H]4[C@@]3(CC5=C(C4)NN=C5)C
169	Steviol	319.2268	173.38	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O)(C)C(=O)O
170	Steviolbioside	643.3324	235.78	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)(C)C(=O)O
171	Stevioside	805.3852	269.64	C[C@@]12CCC[C@@]([C@H]1CC[C@]34[C@H]2CC[C@](C3)(C(=C)C4)O[C@H]5[C@@H]([C@H]([C@@H]([C@H](O5)CO)O)O)O[C@H]6[C@@H]([C@H]([C@@H]([C@H](O6)CO)O)O)O)(C)C(=O)O[C@H]7[C@@H]([C@H]([C@@H]([C@H](O7)CO)O)O)O
172	Sulfachloropyridazine	285.0208	156.90	c1cc(ccc1N)S(=O)(=O)Nc2ccc(nn2)Cl
173	Sulfadiazine	251.0597	148.70	C1=CN=C(N=C1)NS(=O)(=O)C2=CC=C(C=C2)N
174	Sulfadimethoxine	311.0809	166.00	COc1cc(nc(n1)OC)NS(=O)(=O)c2ccc(cc2)N
175	Sulfadimidine	279.0910	159.70	Cc1cc(nc(n1)NS(=O)(=O)c2ccc(cc2)N)C
176	Sulfadoxine	311.0809	166.60	COC1=C(N=CN=C1OC)NS(=O)(=O)C2=CC=C(C=C2)N
177	Sulfamerazine	265.0754	154.80	CC1=NC(=NC=C1)NS(=O)(=O)C2=CC=C(C=C2)N

#	Compound name	Exact mass (*m/z*)	Emperical CCS (Å²)	SMILES
178	Sulfamethizole	271.0318	152.20	Cc1nnc(s1)NS(=O)(=O)c2ccc(cc2)N
179	Sulfamethoxazole	254.0594	146.30	CC1=CC(=NO1)NS(=O)(=O)C2=CC=C(C=C2)N
180	Sulfapyridine	250.0645	142.30	C1=CC=NC(=C1)NS(=O)(=O)C2=CC=C(C=C2)N
181	Sulfaquinoxaline	301.0754	164.80	C1=CC=C2C(=C1)N=CC(=N2)NS(=O)(=O)C3=CC=C(C=C3)N
182	Sulfathiazole	256.0209	148.20	C1=CC(=CC=C1N)S(=O)(=O)NC2=NC=CS2
183	Sulfentrazone	386.9891	173.63	CC1=NN(C(=O)N1C(F)F)C2=CC(=C(C=C2Cl)Cl)NS(=O)(=O)C
184	Sulfisoxazole	268.0750	157.40	Cc1c(noc1NS(=O)(=O)c2ccc(cc2)N)C
185	Tebuconazole	308.1524	167.37	CC(C)(C)C(CCC1=CC=C(C=C1)Cl)(CN2C=NC=N2)O
186	Tebufenpyrad	334.1681	191.25	CCC1=NN(C(=C1Cl)C(=O)NCC2=CC=C(C=C2)C(C)(C)C)C
187	Teflubenzuron	380.9815	171.81	C1=CC(=C(C(=C1)F)C(=O)NC(=O)NC2=CC(=C(C(=C2F)Cl)F)Cl)F
188	Tepraloxydim	342.1467	184.74	CC/C(=N\OC/C=C/Cl)/C1=C(CC(CC1=O)C2CCOCC2)O
189	Terbufos-sulfone	321.0412	172.88	CCOP(=S)(OCC)SCS(=O)(=O)C(C)(C)C
190	Terbuthylazine	230.1167	153.48	CCNC1=NC(=NC(=N1)Cl)NC(C)(C)C
191	Tetraconazole	372.0288	174.34	C1=CC(=C(C=C1Cl)Cl)C(CN2C=NC=N2)COC(C(F)F)(F)F
192	Tetracycline	445.1605	197.30	C[C@@]1([C@H]2C[C@H]3[C@@H](C(=C(C(=O)[C@]3(C(=C2C(=O)C4=C1C=CC=C4O)O)O)C(=O)N)O)N(C)C)O
193	Thiabendazole	202.0433	136.19	n2c1c(cccc1)nc2c3nccs3
194	Thiacloprid	253.0309	154.84	C1CS/C(=N\C#N)/N1CC2=CN=C(C=C2)Cl
195	Thiophanate-methyl	343.0526	174.24	COC(=O)NC(=S)NC1=CC=CC=C1NC(=S)NC(=O)OC
196	Tiamulin	494.3299	218.90	CCN(CC)CCSCC(=O)O[C@@H]1C[C@@]([C@H]([C@@H]([C@@]23CCC(C1([C@@H]2C(=O)CC3)C)C)C)O)(C)C=C
197	Tolfenamic acid	262.0629	153.70	CC1=C(C=CC=C1Cl)NC2=CC=CC=C2C(=O)O
198	Tolmetin	258.1125	156.70	Cc1ccc(cc1)C(=O)c2ccc(n2C)CC(=O)O
199	Trichlorfon	256.9299	136.12	COP(=O)(C(C(Cl)(Cl)Cl)O)OC
200	Triflumuron	359.0405	169.88	C1=CC=C(C(=C1)C(=O)NC(=O)NC2=CC=C(C=C2)OC(F)(F)F)Cl
201	Trinexapac-ethyl	253.1071	152.92	CCOC(=O)C1CC(=O)C(=C(C2CC2)O)C(=O)C1
202	Triticonazole	318.1371	179.18	Clc1ccc(cc1)\C=C2\C(O)(C(CC2)(C)C)Cn3ncnc3
203	Tulathromycin	806.5737	286.40	CCCNC[C@@]1([C@@H](O[C@H](C[C@@]1(C)OC)O[C@H]2[C@@H]([C@H]([C@](C[C@H](CN[C@@H]([C@H]([C@]([C@H](OC(=O)[C@@H]2C)CC)(C)O)O)C)C)(C)O)O[C@H]3[C@@H]([C@H](C[C@H](O3)C)N(C)C)O)C)C)O

#	Compound name	Exact mass (*m/z*)	Emperical CCS ($Å^2$)	SMILES
204	Tylosine	916.5264	332.40	CC[C@@H]1[C@H](/C=C/C(=O)[C@@H]([C[C@@H]([C@@H]([C@@H](CCC(=O)O1)O)C)O)[C@@H]2[C@@H]([C@H]([C@@H]([C@@H](O2)C)O)[C@H]3C[C@@]([C@H](O3)C)(C)O)N(C)C)O)C= O)C)\C)CO[C@H]4[C@@H]([C@@H]([C@H](O4)C)O)O(C)C)OC
205	Valnemulin	565.3670	233.30	C[C@@H]1CC[C@@]23CCC(=O)[C@H]2[C@@]1([C@@H]([C[C@@]([C@@H]([C@@H]3C)O)(C)C=C)OC(=O)C SC(C)(C)CNC(=O)[C@@H](C(C)C)N)C

Table S2. Pesticides spiked at 0.05 mg/kg (50 ppb in extract) to the blind spinach samples (*n*=3). (Note: spiked pesticides were unknown to the analyst)

#	Compound name	Elemental composition	SMILES
1	Ametoctradin	C15H25N5	CCCCCCCCc1c(nc2ncnn2c1N)CC
2	Atrazine	C8H14ClN5	CC/N=c/1\[nH]/c(=N/C(C)C)/[nH]c(n1)Cl
3	Bixafen	C18H12Cl2F3N3O	O=C(c1c(nn(c1)C)C(F)F)Nc3ccc(F)cc3c2ccc(Cl)c(Cl)c2
4	Chlorantraniliprole	C18H14BrCl2N5O2	Cc1cc(cc(c1NC(=O)c2cc(nn2c3c(cccn3)Cl)Br)C(=O)NC)Cl
5	Dinotefuran	C7H14N4O3	CN/C(=N/[N+](=O)[O-])/NCC1CCOC1
6	Flonicamid	C9H6F3N3O	c1cncc(c1C(F)(F)F)/C(=N/CC#N)/O
7	Metaflumizone	C24H16F6N4O2	c1cc(cc(c1)C(F)(F)F)C(=NNC(=O)Nc2ccc(cc2)OC(F)(F)F)Cc3ccc(cc3)C#N
8	Penflufen	C18H24FN3O	O=C(c1c(nn(c1F)C)C)Nc2ccccc2C(C)CC(C)C
9	Pyriofenone	C18H20ClNO5	Cc1cc(c(c(c1C(=O)c2c(c(cnc2OC)Cl)C)OC)OC)OC
10	Valifenalate	C19H27ClN2O5	Clc1ccc(cc1)C(NC(=O)[C@@H](NC(=O)OC(C)C)C(C)C)CC(=O)OC

Table S3. Relative importance (%) of each descriptor to the ANN prediction. (Note: Sum does not equal 100 as it is the mean of 6 replicates)

Molecular Descriptor	Relative importance of each descriptor (%)
Sv	36.7
VEm2	24.6
VEv2	21.1
nR=Cp	7.1
nC	4.7
E1e	2.4
VRD2	0.5
nR09	0.5

Table S4. The 54 molecular descriptors chosen for genetic-algorithm feature selection

Molecular descriptor	Definition
Aeigp	Absolute eigenvalue sum from polarizability weighted distance matrix
AMW	Average molecular weight
ARR	Aromatic ratio
De	Total accessibility index / weighted by atomic Sanderson electronegativities
Depressant-80	Ghose-Viswanadhan-Wendoloski antidepressant at 80% (drug-like index)
E1e*	1st component accessibility directional WHIM index / weighted by atomic Sanderson electronegativities
E2u	2nd component accessibility directional WHIM index / unweighted
E2v	2nd component accessibility directional WHIM index / weighted by atomic van der Waals volumes
Hypertens-80	Ghose-Viswanadhan-Wendoloski antihypertensive at 50% (drug-like index)
Infective-80	Ghose-Viswanadhan-Wendoloski antiinfective at 80% (drug-like index)
Inflammat-80	Ghose-Viswanadhan-Wendoloski antiinflammatory at 80% (drug-like index)
L1e	1st component size directional WHIM index / weighted by atomic Sanderson electronegativities
L1m	1st component size directional WHIM index / weighted by atomic masses
L1s	1st component size directional WHIM index / weighted by atomic electrotopological states
L1v	1st component size directional WHIM index / weighted by atomic van der Waals volumes
LP1	Lovasz-Pelikan index (leading eigenvalue)
Me	Mean atomic Sanderson electronegativity
nAB	Number of aromatic bonds
nBM	Number or mulitpile bonds
nBnz	Number of benzene-like rings

Molecular descriptor	Definition
nC*	Number of carbons
nCconj	Number of conjugated carbon atoms
nCl	Number of chlorine atoms
nCq	Number of total quaternary carbon atoms (sp3)
Neoplastic-50	Ghose-Viswanadhan-Wendoloski antineoplastic at 50% (drug-like index)
Neoplastic-80	Ghose-Viswanadhan-Wendoloski antineoplastic at 80% (drug-like index)
nR=Cp*	Number of terminal primary carbon atoms (sp2)
nR=Ct	Number of aliphatic tertiary carbon atoms (sp2)
nR05	Number of 5-membered rings
nR09*	Number of 9-membered rings
nR10	Number of 10-membered rings
nRCO	Number of ketones (aliphatic)
nS	Number of sulfur atoms
P1v	1st component shape directional WHIM index / weighted by atomic van der Waals volumes
P2u	2nd component shape directional WHIM index / unweighted
Psychotic-50	Ghose-Viswanadhan-Wendoloski antipsychotic at 50% (drug-like index)
RBN	Number of rotatable bonds
Seigp	Eigenvalue sum from polarizability weighted distance matrix
Sv*	Sum of van der Waals forces
Te	Total size index / weighted by atomic Sanderson electronegativities
Tm	Total size/ weighted by atomic masses
Ts	Total size index / weighted by atomic electrotopological states
Tu	Total size index, unweighted
Tv	T total size index / weighted by atomic van der Waals volumes
Ui	Unsaturation index

Molecular descriptor	Definition
VEe1	Eigenvector coefficient sum from electronegativity weighted distance matrix
VEm2*	Average eigenvector coefficient sum from mass weighted distance matrix
VEv2*	Average eigenvector coefficient sum from van der Waals weighted distance matrix
VRD1	Randic-type eigenvector-based index from distance matrix
VRD2*	Average Randic-type eigenvector-based index from distance matrix
VRe2	Average Randic-type eigenvector-based index from electronegativity weighted distance matrix
VRp2	Average Randic-type eigenvector-based index from polarizability weighted distance matrix
X0A	Average connectivity index chi-0
X4A	Average connectivity index chi-4

* The eight molecular descriptors used for the final ANN analysis

For more information on the descriptors, refer to the publications cited within VCClabs (http://www.vcclab.org/lab/indexhlp/).

Table S5. 50th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	1.7	1.6	1.9	1.8
2	1.9	2.0	1.4	1.7
3	1.6	1.6	2.1	1.9
4	1.8	1.8	1.0	3.0
5	1.7	1.8	1.4	2.6
6	2.2	2.2	1.5	2.2
Average	1.8	1.8	1.5	2.2
STD	0.2	0.2	0.4	0.5
CV	10%	13%	27%	23%

Table S6. 60th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	2.2	2.3	2.2	2.5
2	2.3	2.4	1.9	2.5
3	2.1	2.0	2.5	2.5
4	2.5	2.1	1.6	3.6
5	2.2	2.2	1.6	2.9
6	2.6	2.8	2.0	3.2
Average	2.3	2.3	1.9	2.9
STD	0.2	0.3	0.3	0.4
CV	8%	13%	18%	15%

Table S7. 70th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	2.9	3.0	2.7	3.0
2	2.9	3.0	2.4	3.7
3	2.6	2.4	2.7	2.7
4	3.0	2.7	3.0	4.0
5	3.0	3.0	1.8	3.3
6	3.2	3.3	2.3	3.7
Average	2.9	2.9	2.5	3.4
STD	0.2	0.3	0.4	0.5
CV	7%	10%	17%	14%

Table S8. 80th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	3.7	3.8	3.3	3.1
2	3.7	3.6	3.4	4.2
3	3.3	3.3	3.7	3.3
4	3.6	3.4	3.3	4.5
5	3.5	3.6	2.5	3.6
6	3.9	3.9	2.9	4.5
Average	3.6	3.6	3.2	3.9
STD	0.2	0.2	0.4	0.6
CV	5%	6%	13%	16%

Table S9. 85th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	4.0	4.0	3.5	4.1
2	4.3	3.9	4.3	4.4
3	3.8	3.7	4.1	3.8
4	4.0	3.9	3.8	4.9
5	4.0	4.0	3.0	4.7
6	4.6	4.4	4.0	4.8
Average	4.1	4.0	3.8	4.4
STD	0.3	0.2	0.5	0.4
CV	6%	6%	12%	9%

Table S10. 90th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	4.4	4.6	4.0	4.7
2	5.0	5.0	4.7	4.8
3	4.4	4.4	4.7	4.1
4	4.3	4.2	4.3	5.3
5	5.1	5.1	3.9	5.4
6	5.2	5.4	4.6	5.0
Average	4.7	4.8	4.4	4.9
STD	0.4	0.5	0.4	0.5
CV	8%	10%	8%	10%

Table S11. 95th Percentile for all six replicate sets of ANN 8-2-8-1 (in %)

Replicate	All	Training	Validation	Blind Test
1	5.4	5.3	4.1	7.2
2	6.4	6.8	5.7	6.0
3	5.3	5.5	5.0	4.7
4	5.6	5.1	4.9	7.7
5	6.2	6.3	4.9	6.8
6	6.3	6.4	5.1	6.1
Average	5.9	5.9	4.9	6.4
STD	0.5	0.7	0.5	1.0
CV	8%	12%	10%	16%

Table S12. t-Test: Two-Sample Assuming Unequal Variances (99%).

	Empirical CCS	Predicted CCS
Median	174.312537	173.908004
Variance	1235.54681	1239.58272
Observactions	205	205
Hypothesised Mean Difference	0	
df	408	
t stat	0.11642099	
P(T<=t) one-tail	0.45368809	
t-critical (one-tail)	2.33552222	
P(T<=t) two-tail	0.90737618	
t-critical (two-tail)	2.58793292	

Table S13. t-Test: Two-Sample Assuming Unequal Variances (95%)

	Empirical CCS	Predicted CCS
Median	174.312537	173.908004
Variance	1235.54681	1239.58272
Observactions	205	205
Hypothesised Mean Difference	0	
df	408	
t stat	0.11642099	
P(T<=t) one-tail	0.45368809	
t-critical (one-tail)	1.6485969	
P(T<=t) two-tail	0.90737618	
t-critical (two-tail)	1.96579537	

Table S14. Average relative errors (%) of CCS values for the blind prediction of 131 pesticides

Percentile	Average RE (%) for protonated molecules $[M+H]^+$	Average RE (%) for sodium adducts $[M+Na]^+$
50	1.9	4.4
60	2.2	5.3
70	2.4	5.7
80	3.3	6.8
90	4.6	7.6
95	5.3	8.7

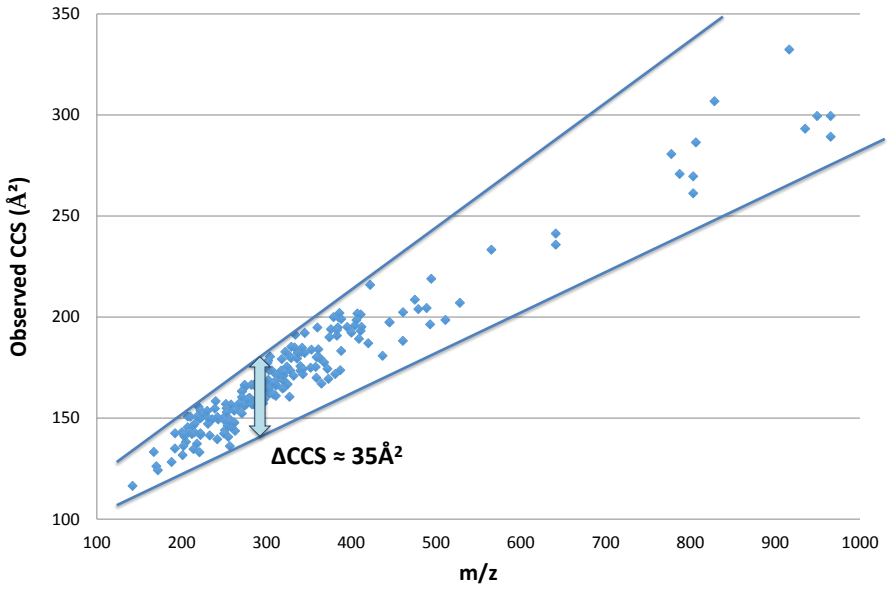

Figure S1. Empirical CCS (Å^2) values *vs.* exact mass (*m/z*) of the protonated molecules of the 205 compounds.

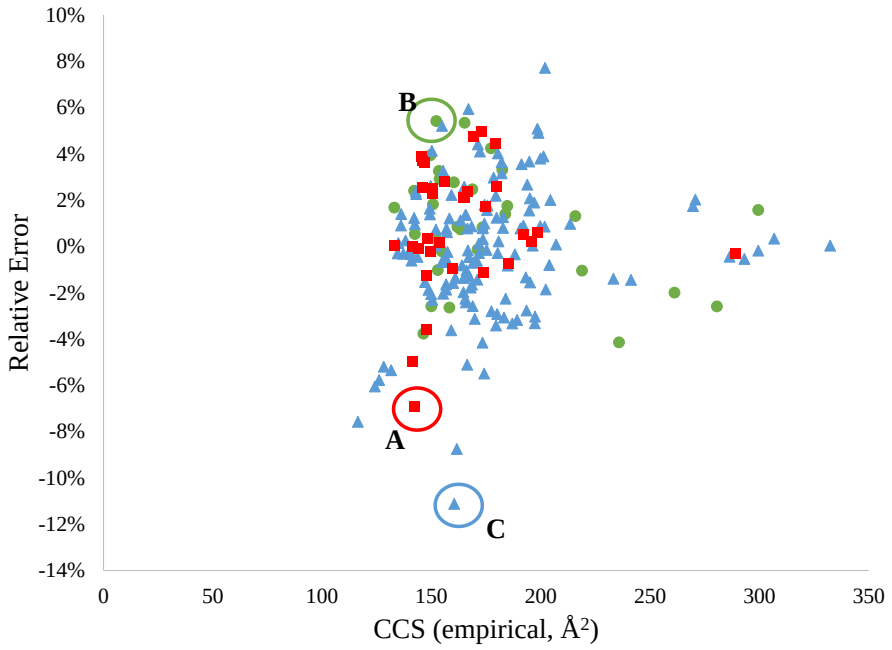

Figure S2. Relative Errors of CCS values for all compounds. Maximum errors for each set is indicated: A: sulfapyridine (-6.9%, validation set), B: chlorbromuron (5.4%, test set), C: acetopromazine (-11.0%, training set). All red squares are the validation set, blue triangles are the training set and green circles are the blind set.

Chapter 3.2.3. Scientific Article 5

DUAL PREDICTION OF RETENTION TIME AND COLLISION CROSS SECTION OF EMERGING ORGANIC CONTAMINANTS FOR ENVIRONMENTAL ANALYSES

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Analytical Chemistry (2021) Submitted

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Keywords

Multivariate Adaptive Regression Splines (MARS); collision cross section prediction; retention time prediction; liquid chromatography; ion mobility separation; high resolution mass spectrometry.

Abstract

Ultra-high performance liquid chromatography coupled to ion mobility separation and high-resolution mass spectrometry instruments have proven very valuable for screening of emerging contaminants in the aquatic environment. However, when applying suspect or non-target approaches (*i.e.* when no reference standards are available) there is no information on retention time (RT) and collision cross section (CCS) values to facilitate identification. *In-silico* prediction tools of RT and CCS can therefore be of great utility to decrease the number of candidates to investigate. In this work, Multiple Adaptive Regression Splines (MARS) was evaluated for the prediction of both RT and CCS. MARS prediction models were developed and validated using a database of 477 protonated molecules, 169 deprotonated molecules and 249 sodium adducts. Multivariate and univariate models were evaluated showing a better fit for univariate models to the empirical data. The RT model (R^2=0.855) showed a deviation between predicted and empirical data of ± 2.32 min (95% confidence intervals). The deviation observed for CCS data of protonated molecules using CCS_H model (R^2=0.966) was ± 4.05 % with 95% confidence intervals. The CCS_H model was also tested for the prediction of deprotonated molecules resulting in deviations below ± 5.86 % for the 95% of the cases. Finally, a third model was

developed for sodium adducts (CCS_{Na}, R^2=0.913) with deviation below ± 5.59 % for the 95% of the cases. The developed models have been incorporated in an open access and user-friendly online platform, which represents a great advantage for third-party research laboratories for predicting both RT and CCS data.

1. Introduction

In the last decade, considerable effort has been devoted to enhance the performance of high resolution mass spectrometry (HRMS) suspect screening (SS) and non-target screening (NTS) strategies [1–3]. The instrumental improvements of HRMS instruments has required the development of more sophisticated algorithms to be able to handle the large amount of data generated [3,4]. Therefore, the development of open-access scripts for data processing and *in-silico* prediction tools represents a step-forward into the applicability of SS and NTS in wide-scope campaigns by facilitating the identification process [5–7]. Furthermore, the establishment of community-adopted levels of confidence for the identification of compounds using chromatography coupled to HRMS has been of paramount importance for the comparison of data across studies [8].

Recently, ion mobility separation (IMS) coupled to HRMS instruments (IMS-HRMS) has proven promising for SS and NTS strategies [9]. It permits, in theory, to resolve co-eluting compounds with same nominal or exact mass that could not be previously separated with solely the chromatographic method, such as isobaric or isomeric compounds [9–11]. Moreover, it allows the removal of mass spectrometric peaks that do not correspond to the feature of interest, which is particularly beneficial in data independent acquisition (DIA) experiments [9,10,12]. As a consequence, there is a reduction in the necessity of data-dependent analysis since the full-spectrum HRMS acquisition can be filtered on both RT and ion mobility data [12,13].

Collision Cross Section (CCS) values, derived from drift time (DT) measured by IMS, are known to be system and matrix-independent and, therefore, empirical CCS data can be included in home-made or online databases with an expected deviation below 2% for most cases [9,14,15]. However, this is not the case for chromatographic retention time (RT) which cannot easily be compared between instrumental configurations. Thus, reference standards are practically required for building a home-made database. However, SS and NTS strategies for the identification of emerging contaminants are commonly applied prior to the acquisition of the corresponding reference standards [1,3] and, therefore,

lacking any information on empirical RT and CCS. In this sense, *in-silico* prediction tools of either chromatographic retention data or ion mobility data are of great utility to decrease the number of candidates to investigate and, therefore, increase the chance of correct identification of features [6].

Several studies have predicted RT [6,16–21], CCS values [7,22–28] or both [13]. Predictors of RT have been developed mainly to model RT data in reverse-phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) with prediction accuracy between approximately ± 1 to ± 3 min. However, there is no clear agreement in the literature on how to express the prediction accuracy of the models or which should be the most appropriate statistical descriptor representing the prediction power of the system developed [6]. Although CCS could be theoretically modelled from the three-dimensional and chemical structure using super-computing systems [27,29–31], data-driven predictive models have also been developed showing predictive accuracies in the range of 3 – 6 % for Travelling Wave Ion Mobility instruments (TWIMS) [22,24,25,28] and Drift Tube Ion Mobility instruments (DTIMS) [23,24,26]. Similar prediction accuracy was obtained by Mollerup *et al.* in their study for the simultaneous prediction of RT and CCS [13]. However, these data-driven models were fed with data generated using different instruments depending on the output parameter. For the RT prediction they used data gathered from an ultra-high performance liquid chromatography (UHPLC)-HRMS instrument, while for CCS prediction they modelled CCS data generated with a UHPLC-IMS-HRMS instrument. Since RT variations could probably be observed across instruments, the utility of predicted RT in the identification of UHPLC-IMS-HRMS features is limited.

In general, the reported models were based on univariate or multivariate regressions [20,28], artificial neural networks (ANNs) [13,18,21,22,24], quantitative structure-retention relationships (QSRR) [6,17,32], supported vector regression (SVR) [23,26] or statistical analysis [25,28]. However, there has been no prior exploration of Multivariate Adaptive Regression Splines (MARS) for the prediction of RT and CCS. MARS is a multivariate non-parametric regression procedure that was first proposed by Friedman [33]. One of the

biggest advantages of MARS compared to the 'black box' methods of ANNs is that they are easy to interpret, with the interactions between variables clearly indicated [34]. MARS has previously been applied in the chemical sciences for quantitative structure-retention relationships [35]. However, the application of MARS for the prediction of chromatographic and ion mobility data of emerging contaminants has not priory been evaluated and reported in the literature.

In this work, a prediction model for both RT and CCS has been developed using MARS for the identification of candidates in SS and NTS strategies using UHPLC-IMS-HRMS. In order to facilitate other laboratories to implement this predictive tool in their workflows, a free online-available application has been released. This is, to best of the authors knowledge, the first application of MARS for the prediction of RT and CCS data. Additionally, it is the first simultaneous RT and CCS predictive model for the same instrument facilitating the identification process of emerging contaminants in SS and NTS strategies.

2. Materials and methods

2.1. Chemicals and materials

A set of 556 reference standards encompassing illicit drugs, hormones, mycotoxins, new psychoactive substances, pesticides and pharmaceuticals were injected for the development of a CCS and RT library [9]. **Table S1** of the **Supporting Information** shows the complete set of compounds used in the study with their SMILES (simplified molecular-input line-entry system) representation, and measured RT and CCS data. This database is also available on the *Zenodo* online repository [36]. Within this dataset, 477 protonated adducts ($[M+H]^+$), 169 deprotonated adducts ($[M-H]^-$) and 249 sodium adducts ($[M+Na]^+$) were used for the development and validation of the CCS predictive models.

2.2. Instrumentation

Retention time and CCS data were obtained with a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) coupled to a VION IMS-QTOF mass spectrometer (Waters, Milford, MA, USA), using an electrospray ionization (ESI) interface operating in positive and negative ionisation mode and following the method presented in Celma *et al.* (2020) [9].

The chromatographic column used was a CORTECS® C18 2.1 x 100 mm, 2.7 µm fused core column (Waters) at a flow rate of 300 µL min^{-1}. Gradient elution was performed using H_2O (A) and MeOH (B) as mobile phases, both with 0.01% formic acid. The percentage of B was initially set to 10%, and it was immediately linearly increased to 90% over 14 min, followed by a 2 min isocratic period, and then returned to initial conditions (at 16.1 min) with a 2 min equilibration of the column. The total run time was 18 min. The injection volume was 5 µL.

A capillary voltage of 0.8 kV and cone voltage of 40 V were used. The desolvation temperature was set to 550 °C, and the source temperature to 120 °C. Nitrogen was used as drying and nebulizing gas. The cone gas flow was 250 L h^{-1} and desolvation gas flow of 1000 L h^{-1}. The column temperature was set to 40 °C and the sample temperature to 10 °C. MS data were acquired using the VION in

HDMSe mode, over the range *m/z* 50-1000, with N_2 as the drift gas, an IMS wave velocity of 250 m s^{-1} and wave height ramp of 20-50 V. Leucine enkephalin (*m/z* 556.27658 and *m/ z* 554.26202) was used for mass correction in positive and negative ionization modes, respectively. Two independent scans with different collision energies were acquired during the run: a collision energy of 6 eV for low energy (LE) and a ramp of 28-56 eV for high energy (HE). A scan time of 0.3 s was set in both LE and HE functions. Nitrogen (≥ 99.999%) was used as collision-induced dissociation (CID) gas. All data were examined using an in-house built accurate mass screening workflow within the UNIFI software (version 1.9.4) from Waters Corporation.

2.3. Retention time and Collision Cross Section Modelling

2.3.1. Molecular descriptors

A total of 1666 molecular descriptors were downloaded from Dragon v5.4 integrated within OChem website (Online Chemical Database with modelling environment, www.ochem.eu) [37]. The complete set of descriptors for the molecules used in the study is available in **Table S1**.

2.3.2. Prediction model

Multivariate Adaptive Regression Splines (MARS) analysis was applied to predict both RT and CCS for protonated adducts ($[M+H]^+$) in a single multivariate model. Additionally, univariate models for individual RT and CCS for protonated adducts ($[M+H]^+$) (CCS_H) and sodium adducts ($[M+Na]^+$) (CCS_{Na}) were also performed. Due to the expected low correlation between RT and CCS (*r=0.354*), a multivariate model was not considered essential. As a further justification for this decision, the cross-validated R^2 values for the multivariate model were 0.798 for RT and 0.964 for CCS_H. This suggests instability on the data that is varying the accuracy of the model fits (particularly for RT). Therefore, the development of a multivariate MARS model able to predict simultaneously RT and CCS simultaneously was discarded.

MARS was able to select the most suitable molecular descriptors for each model (**Table 1**) and predictive interval bands were constructed for the univariate

cases assuming a linear model variance structure. To meet this assumption, the square root of RT was modelled.

The CCS_H prediction model was also explored for the prediction of CCS for deprotonated adducts ($[M-H]^-$) and sodium adducts ($[M+Na]^+$). CCS_H accurately modelled $[M-H]^-$ data, but could not predict data at acceptable levels of accuracy for $[M+Na]^+$. Therefore, a exclusive univariate model was considered for the prediction of CCS data for sodium adducts (CCS_{Na}).

All analyses were complete using R [38] and MARS analysis was completed using the earth package with variance structure defined using the linear model (lm) option [39].

Table 1: Descriptors needed for each of the univariate MARS models for retention time (RT) and collision cross section (CCS_H and CCS_{Na}). Note that there are no similarities between the three univariate models.

Molecular descriptors		
RT	**CCS_H**	**CCS_{Na}**
ALOGP	AMR	AMR
ALOGPS_LogP	L1m	DP01
BEHm4	LPRS	Mor16e
GATS1m	MDDD	Mor17m
Mor16m	nRCHO	Mor27u
N-068	PCR	PW4
nDB	Whetp	QYYm
nRNHR		R4u
O-059		SEigp
STN		TI2

ALOGP: Ghose-Crippen octanol-water partition coefficient (logP) (calculation based on Viswanadhan *et al.* (1989) [40]; *ALOGPS_LogP*: Ghose-Crippen octanol-water partition coefficient (logP) (calculation based on Tetko and Tanchuk (2002) [41]; *AMR*: Ghose-Crippen molar refractivity; *BEHm4*:highest eigenvalue n. 4 of Burden matrix / weighted by atomic masses; *DP01*: molecular profile no. 01; *GATS1m*: Geary autocorrelation - lag 1 / weighted by atomic masses; *L1m*: 1st component size directional WHIM index / weighted by atomic masses; *LPRS*: log of product of row sums; *MDDD*: mean distance degree deviation; *Mor16e*: 3D-MoRSE - signal 16 / weighted by atomic Sanderson electronegativities; *Mor16m*: 3D-MoRSE - signal 16 / weighted by atomic masses; *Mor17m*: 3D-MoRSE - signal 17 / weighted by atomic masses; *Mor27u*: 3D-MoRSE - signal 27 / unweighted; *N-068*: Al3-N atom-centred fragment; *nDB*: number of double bonds; *nRCHO*: number of (aliphatic) aldehydes; *nRNHR*: number of secondary (aliphatic) amines; *O-059*: Al-O-Al atom-centered fragment; *PCR*: ratio of multiple path count over path count; *PW4*: path/walk 4 - Randic shape index; *QYYm*: Qyy COMMA2 value / weighted by atomic masses; *R4u*: R autocorrelation of lag 4 / unweighted; *SEigp*: Eigenvalue sum from polarizability weighted distance matrix; *STN*: spanning tree number (log); *TI2*: second Mohar index TI2; *Whetp*: Wiener-type index from polarizability weighted distance matrix [42].

3. Results and discussion

3.1. Development and validation of prediction models

3.1.1. Individual RT and CCS model development

There is no assumption of an underlying variance structure with the multivariate MARS analysis, and there was no facility to define one within the earth package at the time of implementation. However, for the univariate analyses, a linear model variance structure was defined. This meant the standard deviation was estimated as a function of the predicted response and, hence, allowed for the construction of prediction intervals.

It is essential to use prediction intervals, rather than confidence intervals, in cases where the goal is to predict future values. A prediction interval is wider than a confidence interval and, at the 95% level, will provide bounds within which 95% of predicted values should fall.

All analyses considered the whole set of 1666 molecular descriptors as possible inputs to be used in the models. The assumptions of normality, linearity and homoscedasticity were assessed for the univariate models which held those assumptions. The univariate MARS fit to RT violated the assumptions of linearity and homoscedasticity, so a square root transform was applied. This then reasonably met assumptions.

In summary, three different univariate models were developed for the prediction of RT (**Equation 1**), CCS data for (de)protonated molecules (CCS_H) (**Equation 2**) and CCS data for sodium adducts (CCS_{Na}) (**Equation 3).** As an example and to assist with interpretation, in **eq. 1**, the term 0.099·max(0,(nDB-3)) is equal to 0 for nDB ≤ 3, and equal to 0.099·(nDB-3) for nDB > 3.

The univariate models obtained a cross validated R^2=0.855 for the RT model, R^2=0.966 for the CCS_H model and R^2=0.913 for the CCS_{Na} model. **Table 1** reveals that the univariate models (RT and CCS_H) do not share a single descriptor, lending weight toward the argument that univariate models provide better fits to the data than previously explored multivariate model.

RT model

$$\sqrt{RT} = 2.343 - 0.171 \cdot \max(0, (4.22 - ALOGPS\_logP)) + 0.099 \cdot \max(0, (nDB - 3)) - 0.086 \cdot \max(0, (3 - nDB)) - 0.451 \cdot \max(0, (N.068 - 1)) + 0.725 \cdot \max(0, (1 - N.068)) + 0.632 \cdot \max(0, (1 - nRNHR)) - 2.177 \cdot \max(0, (BEHm4 - 3.582)) - 0.533 \cdot \max(0, (3.582 - BEHm4)) - 1.565 \cdot \max(0, (Mor16m - 0.54)) + 0.111 \cdot \max(0, (ALOGP - 2.719)) - 0.234 \cdot \max(0, (2.719 - ALOGP)) + 0.114 \cdot \max(0, (O.059 - 1)) - 0.138 \cdot \max(0, (1 - O.059)) - 3.185 \cdot \max(0, (GATS1m - 1.422)) - 0.132 \cdot \max(0, (STN - 6.985)) \quad \textbf{(Eq. 1)}$$

CCS_H model

$$CCS_H = 203.344 + 0.482 \cdot \max\big(0, (AMR - 94.347)\big) - 0.524 \cdot \max\big(0, (94.347 - AMR)\big) - 0.002 \cdot \max\big(0, (Whetp - 1940.49)\big) - 0.836 \cdot \max\big(0, (9.95 - L1m)\big) - 14.618 \cdot \max\big(0, (PCR - 1.109)\big) + 36.31 \cdot \max(0, nRCHO) + 0.361 \cdot \max\big(0, (LPRS - 171.967)\big) - 0.157 \cdot \max\big(0, (171.967 - LPRS)\big) - 0.74 \cdot \max\big(0, (28.622 - MDDD)\big) \quad \textbf{(Eq. 2)}$$

CCS_{Na} model

$$CCS_{Na} = 156.001 + 0.471 \cdot \max(0, (AMR - 56.405)) - 0.373 \cdot \max(0, (56.405 - AMR)) + 14.692 \cdot \max(0, (DP01 - 3.94)) + 18.919 \cdot \max(0, (R4u - 2.412)) + 5.025 \cdot \max(0, (Mor16e - 0.031)) + 0.583 \cdot \max(0, (SEigp - 11.443)) + 2.264 \cdot \max(0, (-11.443 - SEigp)) - 3.298 \cdot \max(0, (TI2 - 2.068)) - 8.14 \cdot \max(0, (2.068 - TI2)) + 0.009 \cdot \max(0, (QYYm - 357.711)) - 0.047 \cdot \max(0, (357.711 - \mathrm{QYYm})) + 7.098 \cdot \max(0, (Mor27u - 0.121)) + 7.027 \cdot \max(0, (Mor17m - 0.293)) + 6.533 \cdot \max(0, (-0.293 - Mor17m)) + 139.123 \cdot \max(0, (PW4 - 0.171)) \quad \textbf{(Eq. 3)}$$

3.1.2. RT, CCS_H and CCS_{Na} model validation

MARS models were fit using a 3-fold cross validation with thirty iterations. This procedure splits the data into three sections, fits the model to two of those sections (*training data*) and then tests the accuracy of the resulting model on the final section (*test data*). This procedure is then repeated thirty times, each time randomly dividing the data into three sections. The measure of accuracy used to assess goodness of fit is the cross-validated R^2, which looks at the average R^2 value obtained across all thirty iterations when the model was fit to the test data. This value is usually lower than the R^2 for the best model fit but dramatic changes suggest volatility in the data or overfitting in the modelling procedure.

In order to perform an additional model validation and to obtain an overview of the model performance, RT and CCS data was predicted for the molecules used for model development. By comparing predicted and empirical RT data (**Figure 1A, top**), it was observed that the average deviation obtained using RT model (**eq. 1**) was ± 0.72 min as shown in **Table 2**. Yet, 95% of the predictions fell within ± 2.32 min. Additionally, it could also be observed that deviations in predicted data distributed normally around 0% deviation (marked as a red line in **Figure 1A, bottom**). The prediction accuracy obtained is an improvement for the 95% intervals in previously developed models (± 4.0 min using *logKow* predictor [20], ± 2.80 min using ANNs [21]) and in line with the model developed by means of ANN by Mollerup *et al.* (over ± 2 min deviation) [13]. The developed model herein presented also improves the prediction accuracy compared to Barron *et al.* where they obtained average deviation of ± 1.02 min [18]. As another way of presenting prediction accuracy, **Figure 2** plots the predicted vs. empirical data with the 95% prediction intervals (soft blue) for the univariate MARS analysis of the $\sqrt{\text{RT}}$. Approximately, only 8% of predicted RT were more than 2 min away from empirical ones.

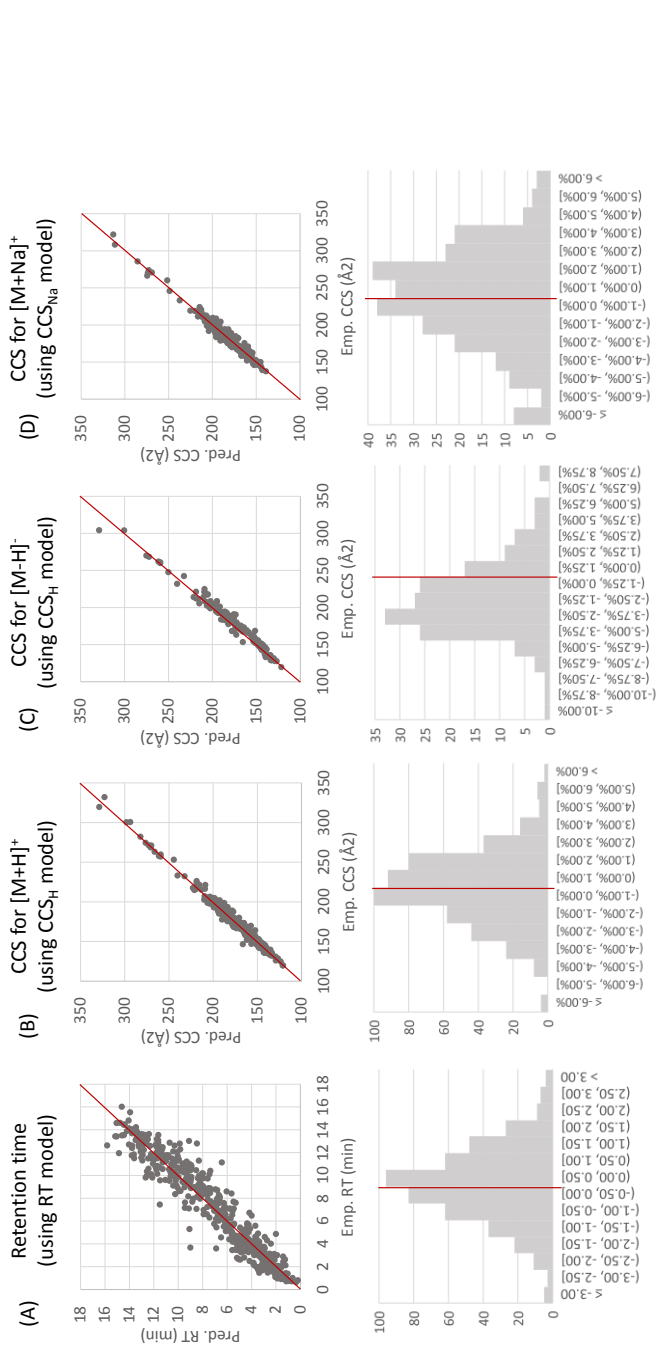

Figure 1. *Top:* Comparison of empirical and predicted RT data using RT model (A), CCS for protonated molecules using CCS_H model (B), CCS for deprotonated molecules using CCS_H model (C), and CCS for sodium adducts using CCS_{Na} model (D). (Red line indicates region where Empirical CCS = Predicted CCS) *Bottom:* Histogram distribution of deviations between empirical and predicted data for RT data using RT model (A), CCS for protonated molecules using CCS_H model (B), CCS for deprotonated molecules using CCS_H model (C), and CCS for sodium adducts using CCS_{Na} model (D). (Red vertical lines indicates 0% deviation)

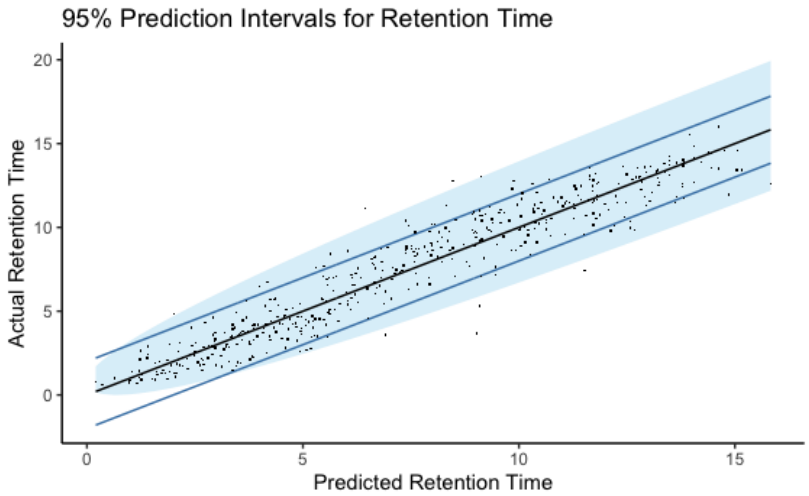

Figure 2: 95% prediction intervals (soft blue) for the univariate MARS analysis on the square root of RT. The blue lines are placed at the predicted values ± 2 min. Approximately, only 8% of observed retention times were more than 2 minutes away from their predicted value.

Table 2. Deviations at percentiles 50 (average), 95 and 99 for the predicted RT and CCS data during model validation.

Model		Average deviation	Deviation at 95%	Deviation at 99%
RT		± 0.72 min	± 2.32 min	± 3.82 min
CCS_H	**$[M+H]^+$**	± 1.23 %	± 4.05 %	± 6.33 %
	$[M-H]^-$	± 2.79 %	± 5.86 %	± 8.39 %
	$[M+Na]^+$	± 4.77 %	± 10.86 %	± 12.80 %
CCS_{Na}	**$[M+Na]^+$**	± 2.13 %	± 5.59 %	± 6.68 %

Prediction accuracy for CCS data was also studied. The deviation observed for CCS data of $[M+H]^+$ using CCS_H model averaged ± 1.23 %, being ± 4.05 % within 95% of the cases (**Table 2**). **Figure 1B,** bottom shows that deviations randomly distributed around 0% (marked as a red line) value without biasing predicted data. When compared with previous models, CCS data for protonated molecules could be predicted using ANNs with an accuracy of ± 5 – 6% for 95% of the cases [13,22] or slightly over ± 5 % deviation (95% confidence interval) using machine

learning [25]. **Figure 3A** shows the 95% prediction intervals (soft blue) for the univariate MARS analysis on CCS_H model. The blue lines are placed at (predicted values ± 2 Å^2) and the purple are ± 5 Å^2. It is clear that the model is still predicting well at higher values where there is less data but the prediction intervals are much larger to accommodate the uncertainty due to lack of data. This vast improvement in the accuracy could be explained because of the larger database used for the model development as well as the better fitting of empirical data with MARS than ANNs.

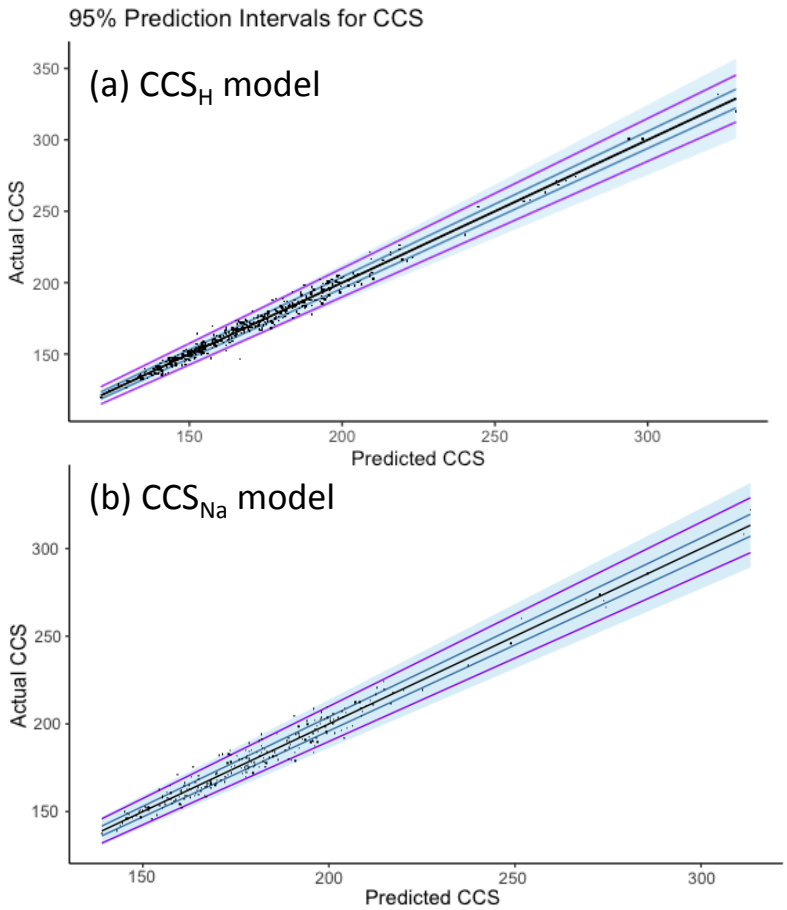

Figure 3: 95% prediction intervals (soft blue) for the univariate MARS analysis on (a) CCS_H and (b) CCS_{Na} models. The blue lines are placed at (predicted values ± 2 Å^2) and the purple are ± 5 Å^2. It is clear that the model is still predicting well at higher values where there is less data but the prediction intervals are much larger to accommodate the uncertainty due to lack of data.

Additionally, generalisability of the CCS_H model for the prediction of CCS values for deprotonated molecules was tested, yielding highly accurate predictions (**Figure 1C, top**). By predicting mobility data for a set of 169 molecules ionized in negative mode, it was observed that the differences between the observed and predicted CCS for the $[M-H]^-$ fell, 95% of the time, within -13.4 and 9.3 $Å^2$, with a slight tendency to under-predict CCS values (**Figure 1C, bottom**). In relative terms, average deviation for $[M-H]^-$ data was ± 2.79 % (± 5.86% for the 95% of the cases, **Table 2**). Although these deviations seem larger than the ones observed for $[M+H]^+$ data, this increase in the deviations observed for $[M-H]^-$ was anticipated since it responds to the fact that the model was developed with $[M+H]^+$ data. However, it was assumed that the predictions of CCS_H model developed with $[M+H]^+$ data could also be extrapolated to the prediction of CCS data for $[M-H]^-$, since no remarkable improvement was expected if a model was exclusively developed for deprotonated molecules.

Ideally, a unique model for the prediction of CCS for (de)protonated molecules and sodium adducts was intended. Therefore, generalisability of the CCS_H model was also tested against $[M+Na]^+$ data. However, high deviations were observed (± 4.77 % average, ± 10.86 % for the 95% of the cases, **Table 2**) as it could be expected due to the likely higher impact of the volume of the sodium atom in the overall CCS of the molecule. In light of this data, $[M+Na]^+$ data required a separate model for CCS prediction that was different to the one initially developed. The procedure for CCS_{Na} model development is equivalent to the process described above but using as input a dataset of 249 CCS values for $[M+Na]^+$ ions. The accuracy of the model was evaluated by also comparing predicted and empirical data (**Table 2**). Prediction deviations were ± 2.13 % on average (± 5.59 % for the 95% of the cases) showing a great improvement compared with predicted data using the CCS_H model. The fact that different predicted values can be obtained for both protonated molecules and sodium adducts is of great help for empirical observations of both species for a suspect substance. Hence, increased confidence on the tentative identification can be garnered by matching both of the CCS values observed with predicted data.

The CCS_{Na} model herein presented also improves the prediction accuracy of previously developed model by the authors [22]. In that work, we evaluated the performance of the ANN predictive model for sodium adducts finding that deviations between predicted and empirical data were below 8.7% for the 95% of the cases. However, the development of an exclusive model for the sodium adducts by MARS improves the prediction accuracy.3.3. Performance of the CCS Predictor

3.2. Open access prediction platform

To aid future researchers working with UHPLC-IMS-HRMS, a free online webpage incorporating these models has been released. The models are available for the scientific community through https://melsapps.shinyapps.io/Predicting_CCS_and_RT/. **Figure 4** illustrates the layout of the online platform for the prediction of RT and CCS for both (de)protonated molecules or sodium adducts.

The operational of the platform is user-friendly and easy-to-follow. As an example, the step-by-step method to obtain prediction for omeprazole is shown. First, selection of which parameter is going to be predicted need to be done (**Figure 4A**). In this case, CCS for protonated molecule is selected by indicating 'Select Response: Collision Cross Section' and 'Sodiated: No'. After downloading the appropriate descriptors for the molecule of interest using Dragon v5.4 integrated within OChem (www.ochem.eu) [37], those can be added in the corresponding editable fields (**Figure 4B**). The CCS value can, then, be predicted and the output is shown together with their corresponding prediction intervals (**Figure 4C**). In this case, the CCS predicted value for the protonated molecule of omeprazole is 181.51 $Å^2$ with a prediction interval of 171.93 – 190.08 $Å^2$. The empirical value for $[M+H]^+$ for omeprazole is 180.58 $Å^2$, denoting that the prediction only deviated 0.52% from the empirical value.

The ease of prediction as well as the open access for this online platform is of great help for those researchers working on UHPLC-IMS-HRMS instruments who do not have an in-house developed prediction model.

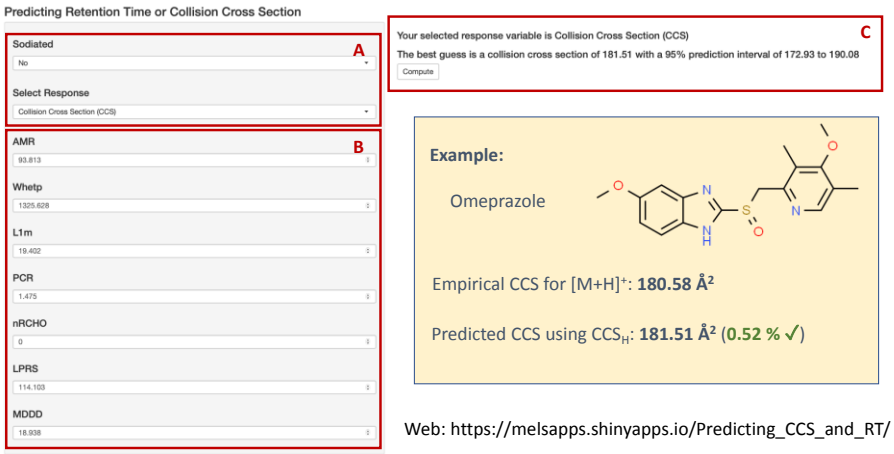

Figure 4. Online platform for the prediction of RT and CCS data using univariate models. (A) Selection of response to predict *i.e.* RT, CCS for (de)protonated molecules or CCS for sodium adducts; (B) Introduction molecular descriptors for the molecule of interest; (C) Output of the predictor model together with the prediction intervals. Example illustrated by omeprazole.

4. Conclusions

Three different prediction models using Multiple Adaptive Regression Splines have been developed for the prediction of RT, CCS for (de)protonated molecules and CCS for sodium adducts. This is the first application of MARS for the prediction of RT and CCS data. In addition, the reported models are the first parallel prediction of RT and CCS data for the same instrument, facilitating the identification process of chemicals of emerging concern in SS and NTS strategies. The developed predictive models make use of a set of 26 molecular descriptors to predict RT and/or CCS values. The prediction accuracy achieved with these models bettered previously reported models in the literature by reducing the deviation between predicted and empirical to ± 2.32 min for RT, ± 4.05 % for CCS of protonated molecules, ± 5.86 % for CCS of deprotonated molecules and ± 5.59 % for CCS of sodium adducts (95% confidence intervals). Additionally, a free access online platform has been released to enable the application of these models to third-party laboratories interested in predicting RT and CCS data.

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Supporting Information

Table S1 of the **Supporting Information** shows the complete set of compounds used in the study with their SMILES (simplified molecular-input line-entry system) representation, empirical RT and CCS data as well as the whole set of descriptors used for model development.

Due to space limitations, this table is available upon request.

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3.3. Discussion

In this chapter, different methodologies towards the refinement of suspect and non-target analysis have been presented. While **scientific article 3** presented a RT interpolation system, **scientific articles 4** and **5** showed different approaches for the *in-silico* prediction of RT and/or CCS. These topics herein tackled received much attention in the suspect and non-target screening fields with different studies being recently published (Hollender et al., 2017; Menger et al., 2020; Miller et al., 2018; Pourchet et al., 2020; Rigano et al., 2021; Ross et al., 2020). The benefits of implementing such type of strategies for the screening of chemicals have been well evaluated elsewhere (Bade et al., 2015a; Bijlsma et al., 2019). When developing refinement tools such as RTI or predictive models there are several key points for a proper implementation that need to be individually addressed.

Retention time indexing approaches. Added value and key elements.

Retention time agreement between measurements in samples and reference standards is key for an appropriate identification of candidates (Hernández et al., 2019; Menger et al., 2020; Schymanski et al., 2014a). However, it is also well

known and demonstrated in **scientific article 3**, that compound retention can be strongly affected by matrix components, changes in mobile phase, etc. Thus, RTI strategies can provide a robust, reliable, matrix-independent and system-independent parameter for comparison purposes. Some of the main aspects for a successful RTI strategy can be summarized as follows:

i. ***Coverage of the whole chromatographic run.*** The selection of appropriate markers for the RTI system should be strategically considered as it is one of the main challenges of RTI for LC (Rigano et al., 2021). Different approaches have selected series of compounds with increasing levels of polarity, such as *n*-nitroalkanes (Hall et al., 2012) so that markers are spread over the chromatographic run. However, they might not cover the whole range of polarities and/or do not suffer from the same retention distortions as analytes of interest. Therefore, the selection of markers covering the entire chromatographic run, from the very first compound eluted in the dead volume to the very last compound eluted at the end of the chromatographic gradient, is pivotal. In **scientific article 3**, efforts were devoted to select appropriate compounds. Hence, ecgonine-d_3 was selected as the initial marker since it was eluted with the dead volume. Similarly, DEHP-d_4 (RTi-P) and THC-COOH-d_3 (RTi-N) were selected as the last markers since no other peak was observed afterwards or no other isotopically labelled reference standard eluting afterwards was available in the laboratory. Thus, the whole chromatographic run is covered by RTi markers. Although no series of homologue compounds were used, appropriate distribution and coverage of the whole chromatographic space was achieved.

ii. ***Compound similarity.*** Having similar chemical properties between target or suspect analytes and markers is necessary so that the retention behavior can change in the same way. However, the distinct factors affecting compound retention (polarity, water solubility, etc.) and the wide range of LC-amenable compounds represent the biggest challenges for RTI strategies. In the end, most of the developed strategies rely on reference compounds available in the laboratory and, therefore,

similarity across markers and analyte chemical families is expected. As an example, the RTi presented in **scientific article 3** was developed by means of different standards available in our laboratory covering the different chemical families most often studied in our institute (pesticides, pharmaceuticals, illicit drugs, new psychoactive substances, etc.). However, this limits the finding of a universal LC-RTI strategy useful for the analysis of whatever LC-amenable compound in whichever system.

iii. ***Easiness of marker differentiation.*** The co-injection of standards is preferred for RTI systems. Therefore, markers should be easily differentiated from matrix endogenous compounds to avoid the reporting of false negatives. Therefore, the utilization of isotopically labelled compounds as proposed in **scientific article 3** is strongly recommended. These added standards share the same properties as the non-labelled homologues and, consequently, will be affected in the same manner by changes in chromatography. Additionally, they can be easily differentiated by means of MS and, therefore, limit the reporting of false negatives.

iv. ***Inter-system applicability.*** Ideally, an RTI approach should be able not only to compensate for chromatographic changes but also for changes in the chromatographic system such as column length, mobile phase composition, stationary phase, column particle size, etc. However, the elevated number of factors influencing the compound retention complicates the finding of an appropriate strategy. In **scientific article 3**, the developed RTi was evaluated by applying it using two different chromatographic columns. The observations led to the conclusion that the interpolation system needs to be slightly adapted to be used with different columns. However, it was demonstrated that the approach was robust enough to correct for RT deviations due to matrix effect (**Table 3** of **scientific article 3**). However, more efforts are needed to find a better set of markers that can be applied over more and different chromatographic systems.

v. ***Wide implementation.*** A successful RTI approach needs to be widely accepted and implemented among the scientific community. The consequent creation of online available databases of RTI values for compounds of interest would represent a milestone for the refinement of retention measurement in LC analyses. So far, there has been no such acceptance over the different RTI strategies reported since a universal series of markers could not be found. The wide range of chemical properties of LC-amenable compounds translates into extremely different chromatographic behavior and, therefore, marker compounds need to be selected based on the intended application (Rigano et al., 2021).

vi. ***Signal intensity.*** Marker compounds should be selected based on their response in the MS detector. Preferably, markers should have similar sensitivity and are spiked at concentrations in the same level of compound of interest. The reason for this is that otherwise markers may mask the signal of a naturally occurring compound.

vii. ***Cost and marker stability.*** The cost and stability of markers should always be taken into account. Although isotopically labelled compounds are preferred (easiness to differentiate from naturally occurring chemicals), their elevated cost, and potential stability issues, may represent a limitation.

There are several key points to be considered for the development of an appropriate RTI strategy. Nevertheless, it is also expected that the future of retention measurement in LC will be based on a universal indexing system as it is the case for GC. The published studies presented in this chapter lay the basis for future studies to develop this universal strategy.

Implementation of *in-silico* prediction tools for suspect and non-target analyses

The increasing trend to perform large suspect and/or non-target screenings pushed towards the development of *in-silico* prediction tools for mass

fragmentation, RT and CCS. Among them, mass fragmentation predictive models (Ruttkies et al., 2016; Wolf et al., 2010) are the most widely spread and implemented for environmental analysis. Yet, the number of publications dealing with the prediction of RT and/or CCS is also raising with many different technologies being evaluated such as ANN, supported vector machine, etc. (Miller et al., 2018). As shown in **scientific articles 4** and **5**, the attention within this thesis has been devoted to RT and CCS prediction models.

For the development of suitable prediction models, there are different aspects that need to be considered:

i. ***Coverage of different chemical families.*** The performance of prediction models relies, among other factors, on the number of empirical data used for their development and validation. Thus, the utilization of large and curated databases is preferred. In this chapter, **scientific article 4** presented a CCS prediction model developed using 205 CCS values for protonated molecules. In order to expand the applicability of CCS prediction, a new model was developed in **scientific article 5** with information of 556 reference standards (including (de)protonated molecules and sodium adducts). The latter covered a wider range of chemical compounds and families and, therefore, more chemical domains were considered within the model.

ii. ***Improvement in prediction accuracy.*** The currently available prediction models for both RT and CCS data are still exceeding the commonly applied deviation thresholds for empirical data. While RT and CCS are often limited to 0.1 min or 2% deviation from standards, respectively, prediction models will not reach that accuracy in their predictions. Nevertheless, efforts need to be devoted to develop more accurate and meaningful models. In this chapter, prediction accuracy for CCS values was increased in **scientific article 5** compared to **scientific article 4** by reducing the deviations observed in predicted CCS values from 6% to 4%. For RT, previous models developed within the research group (Bade et al., 2015a, 2015b) were also upgraded by reducing the predicted RT deviation by 0.5 min. Although the developed models do

not yet reach the ideal prediction accuracy levels, it is expected that future developments as well as larger databases and/or alternative modelling approaches will meet those requirements.

iii. ***System (in)dependency.*** Ideally, predictive tools for RT and CCS should be valid across different instruments or systems; therefore, there would be no need to re-train or adapt existing models to third-party laboratories. However, RT prediction models cannot be straightforwardly used to predict retention data in other systems because of the nature of retention in LC. For CCS values, which can be compared between different instruments, prediction models could also be applied to measurements observed in different systems (Ross et al., 2020; Zhou et al., 2020). Yet, there are no available studies evaluating the accuracy of models when predicting data for an instrument that uses a different technology than the one used for the model construction.

iv. ***Model accessibility.*** One of the biggest challenges for prediction tools is being accessible for the whole scientific community. Although in-house prediction tools are very useful, developing such tools requires sophisticated software, high background knowledge on statistics as well as large amounts of reference standards to train the model. Yet, not all laboratories can have the infrastructure to generate their own predictive tools. Therefore, it is necessary that prediction models are available to third-party laboratories through online applications to calculate RT and CCS. In this context, predictive models for RT and CCS developed in **scientific article 5** has been publicly released through a free-access webpage. With the online available prediction model, interested researchers can calculate RT and CCS with simply typing the SMILES of the compound of interest.

Future perspectives for refinement tools in suspect and non-target analysis

In the forthcoming years, it is expected that several refinement tools for an enhanced performance of suspect and non-target screening approaches will be developed. Improvements in the accuracy of predictive models for RT, CCS and

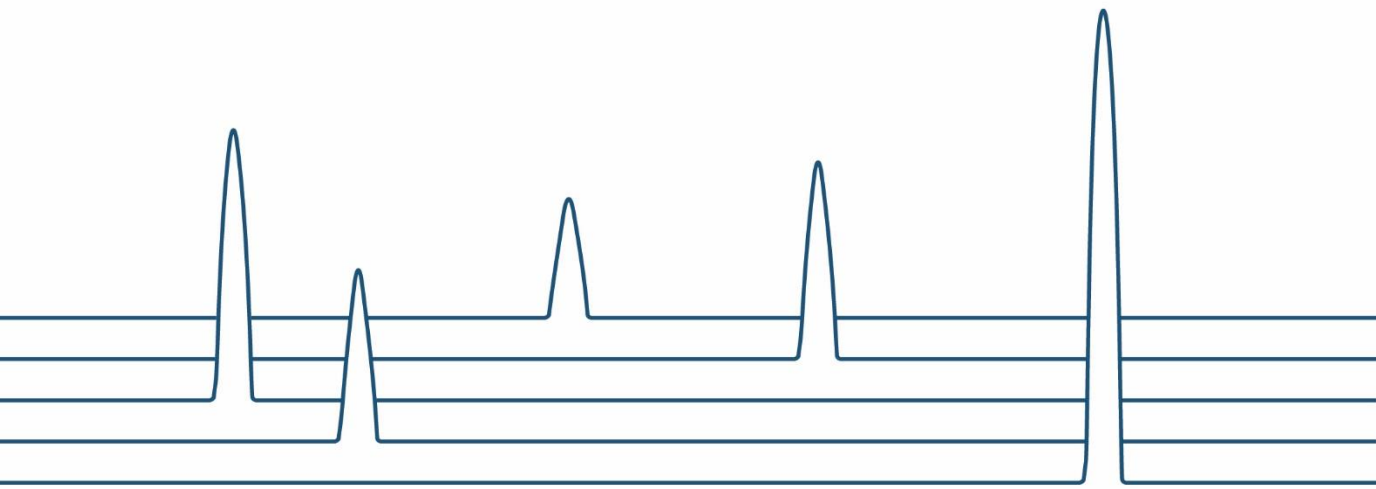
also mass fragmentation will be achieved by the betterment of the models and/or alternative statistical analysis.

In the particular field of retention measurement, the finding and adoption of a universal RTI strategy for LC system will represent a milestone for the field of environmental analyses. Afterwards, the development of prediction tools for RTI will be essential in order to harvest the greatest benefits of an accurate relative measurement of RT for suspect and non-target approaches.

Additionally, improvements in accuracy for CCS predictive models are also expected due to the increase of available CCS values. Moreover, smart CCS predictive models able to foresee which ionization mode and adducts can be formed by the compound of interest and, therefore, a predicted CCS value for that particular adduct will improve the comprehensiveness and quality of the predictions.

CHAPTER 4.

THE USE OF TOXICOLOGICAL FINGERPRINTS AS A TOOL FOR A REFINED SCREENING STRATEGY



Chapter 4. The use of toxicological fingerprints as a tool for a refined screening strategy

4.1. Introduction

4.2. Scientific Articles

4.2.1. Scientific article 6.

"*In vitro* bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline"

Environmental Sciences Europe 33 70 (2021) 1-12

4.2.2. Scientific article 7.

"Integration of chemical analysis with bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies"

Water Research (2021) Submitted

4.3. Discussion of Results

4.1. Introduction

The combined utilization of TS, SS and NTS together with *in-silico* prediction tools and RTI strategies for the assessment of fate of OMPs in the environment has been proven a powerful strategy (Hernández et al., 2019; Hollender et al., 2017; Menger et al., 2020; Schymanski et al., 2015). However, only a minuscule fraction of the chemicals present in environmental matrices are often identified under these strategies (Hollender et al., 2017). Chemical analysis of environmental samples might be limited by several factors such as the selection of appropriate chromatographic separation and/or mass spectrometric analysis, among others. LC coupled to MS is often the technique of choice to analyze aquatic environmental samples because of the associated ease to analyze aqueous matrices. Thus, only a limited space of the whole chemical universe can be monitored (**Figure 4.1**). Conversely, various studies have also used GC for the environmental monitoring of OMPs or even the combination of both LC and GC over the same samples to widen the chemical space assessed (Hernández et al., 2015). However, chemical analysis cannot cover every single substance present in the sample nor it does provide information on potential adverse biological effects of present compounds on the surrounding organisms.

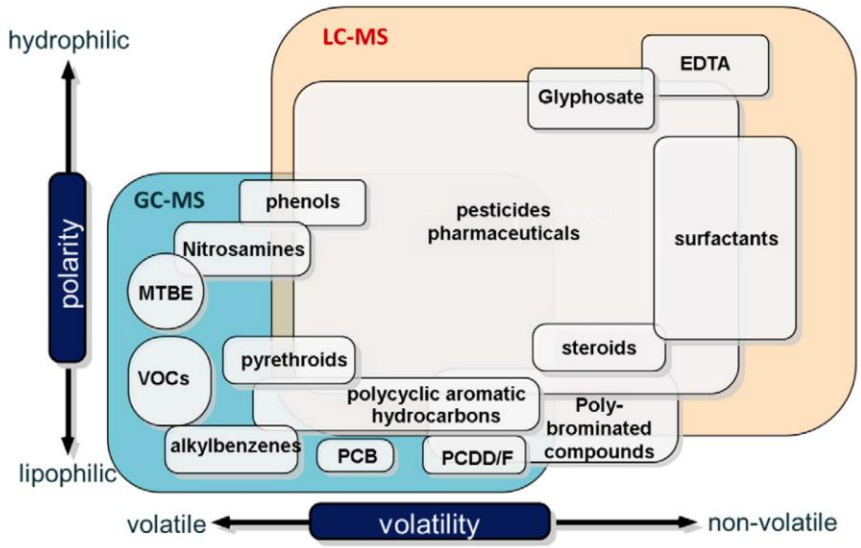


Figure 4.1. Domains of LC-amenable and GC-amenable compounds when analyzed by chromatographic separation coupled to MS. (Extracted from Brack et al., 2016).

Biological analysis of samples can, therefore, be of help to overcome such limitation by providing an overall insight on the toxicity of the sample for certain endpoints. Cell-based bioanalysis provide a measure of mechanisms of toxicity by picturing the interaction of stressors (*e.g.* OMPs) with certain biological targets (Escher et al., 2014). In other words, a bioassay can be defined as an estimation of the potency of a physical, chemical or biological agent by measuring and comparing the magnitude of the cellular response with that of a known standard (Panuganti, 2015). Those studies can be performed *in vivo*, meaning the utilization of alive pluricellular organisms, or *in vitro*, which uses isolated tissues for the analysis. Both types of bioassays imply the application of an external stimulus (or stressor) to a subject (biological organisms) which is often followed by a change in some measurable characteristics of the subject. Thus, a relation between the magnitude of the change and the actual amount of stressor applied can be observed (Panuganti, 2015).

Numerous bioassay methodologies are available in the scientific literature for different toxicity endpoints, samples as well as distinct *in vivo/in vitro* organisms. However, the range of bioanalytical tools developed for the analysis

of environmental aquatic samples is more restricted; mainly, due to the specific toxicity endpoints relevant to be monitored in the environment (Escher et al., 2014).

Bioanalysis of toxicological fingerprint of aquatic samples is a powerful tool to assess the quality of environmental matrices. However, *in vitro* bioassays alone are not able to comprehensively assess water quality (Dopp et al., 2019; Escher et al., 2014). Thus, the combination of chemical analysis with biological analysis is a holistic approach that aims to cover an as wide as possible range of chemicals and potential toxic effects detected in the ecosystems. To this purpose, effect-directed analysis (EDA) has revolutionized the monitoring of OMPs in the aquatic environment by both identifying chemicals and their associated risk for the surrounding organisms (Hernández et al., 2019). EDA is an iterative approach which uses bioassay data in combination with sample fractionation to narrow down the list of potential substances to further evaluate and identify in a toxic sample (Dopp et al., 2019). As depicted in **Figure 4.2,** bioanalysis of samples by EDA is followed by fractionation and chemical analysis of bioanalytically relevant fractions. This sequence can be successively applied until bioactive chemicals in complex matrices are identified.

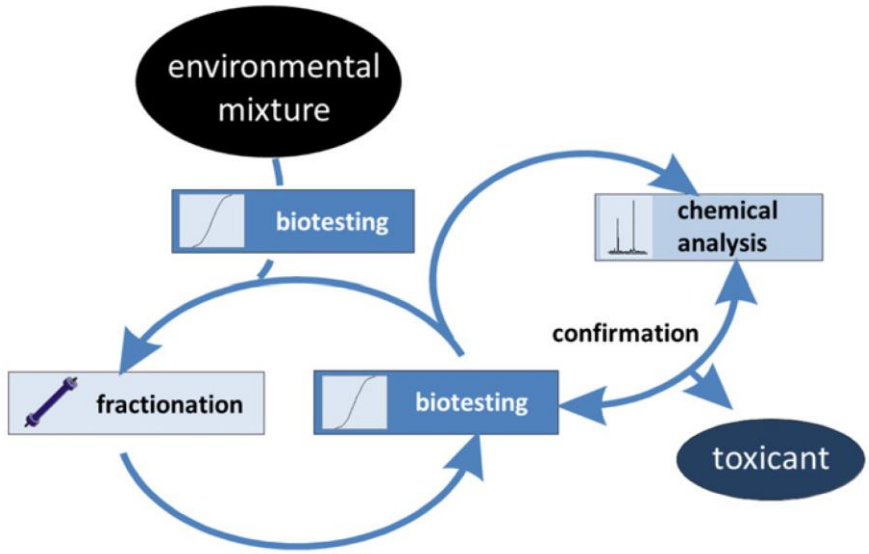


Figure 4.2. General scheme of Effect-directed analysis workflow. (Extracted from Brack et al., 2016).

Although EDA is very promising, only few studies have been able to complete authentic EDA applications due to the intrinsic difficulties and the high level of instrumental sophistication required. Recently, a real EDA approach developed by Tian *et al.* caught much scientific attention due to the discovery of a previously unreported toxic CEC responsible for unprecedented rates of death among fish in small creeks (Tian et al., 2021). By means of combined bioanalysis and fractionation using different separation column chemistries, authors were able to isolate the toxic fraction of the sample. Afterwards, chemical analysis permitted the identification and confirmation of a quinone TP of *N*-(1,3-dimethylbutyl)-*N*′-phenyl-*p*-phenylenediamine (6PPD) originating from the wear of vehicle rubber tires.

While this is a perfect example of EDA combined with NTS, the vast majority of studies using both biological and chemical analyses are devoted to the assessment of specific group of chemicals, either in TS or SS, and link the observed toxicity with the chemicals detected (Brunner et al., 2020; Lundqvist et al., 2019b; Tousova et al., 2017). Whereas these might not meet the requirements of a comprehensive EDA approach, such studies represent a step-forward into the progressive implementation of EDA strategies as a water quality monitoring tool.

4.2. Scientific articles

In this chapter, two different studies have been conducted to monitor quality of Spanish Mediterranean coastline water bodies by the combination of biological and chemical analyses. The articles herein presented have been developed within the framework of the DANTE project "Effect-directed analysis as a tool towards a non-toxic environment - identification of mixture effects and toxicity drivers in water" financed by Swedish Research Council (FORMAS) (project nr. 2018-02256). Furthermore, these studies resulted from an international pre-doctoral research stay at the Swedish University of Agricultural Sciences (SLU) in Uppsala (Sweden) under the supervision of Dr. Johan Lundqvist, Dr. Lutz Ahrens and Prof. Karin Wiberg.

The first work (**scientific article 6**) presents the bioanalysis of 11 surface water samples collected along the Spanish Mediterranean coastline. To this purpose, a panel of 8 different bioassays were applied. The toxicity endpoints evaluated were selected based on their significance for the monitoring of environmental samples and their potential to detect effects from mixed OMPs in surface water samples.

Then, chemical analyses were performed over the same sample set and presented in **scientific article 7**. In this study, a large TS and SS covering different OMP families was conducted by both UHPLC-IMS-HRMS and UHPLC-LRMS. Additionally, the OMPs identified in the sample were compared with observed bioactivities previously reported in **scientific article 6** to integrate both strategies into a more comprehensive approach. To this purpose, *in silico* prediction of compound toxicity was also applied.

In combination, both articles represent a starting point for the development of EDA studies in surface water samples within the framework of DANTE project.

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

- *Environmental Sciences Europe 33 70 (2021) 1-12*
- *Water Research (2021) Submitted*

Chapter 4.2.1. Scientific Article 6

IN VITRO BIOANALYTICAL ASSESSMENT OF TOXICITY OF WETLAND SAMPLES FROM SPANISH MEDITERRANEAN COASTLINE

Alberto Celma, Geeta Mandava, Agneta Oskarsson, Juan Vicente Sancho, Lubertus Bijlsma and Johan Lundqvist

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Environmental Sciences Europe

RESEARCH Open Access

In vitro bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline

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Keywords

Surface water; In vitro bioassay; Aryl hydrocarbon receptor; Androgen receptor; Estrogen receptor; Oxidative stress; Nrf2; Vitamin D receptor; Coastal lagoon; TCDD-eq.

Abstract

Background: Fresh water bodies represent less than 1% of overall amount of water on earth and ensuring their quality and sustainability is pivotal. Although several campaigns have been performed to monitor the occurrence of micropollutants by means of chemical analysis, this might not cover the whole set of chemicals present in the sample nor the potential toxic effects of mixtures of natural and anthropogenic chemicals. In this sense, by selecting relevant toxicity endpoints when performing in vitro bioanalysis, effect-based methodologies can be of help to perform a comprehensive assessment of water quality and reveal biological activities relevant to adverse health effects. However, no prior bioanalytical study was performed in wetland water samples from the Spanish Mediterranean coastline.

Methods: Eleven samples from relevant water bodies from the Spanish Mediterranean coastline were collected to monitor water quality on 8 toxicity endpoints. Aryl hydrocarbon receptor (AhR), androgenicity (AR+ and AR−),

estrogenicity (ER+ and ER−), oxidative stress response (Nrf2) and vitamin D receptor (VDR+ and VDR−) reporter gene assays were evaluated.

Results: AhR was the reporter gene assay showing a more frequent response over the set of samples (activated by 9 out of 11 samples), with TCDD-eq in the range 7.7–22.2 pM. For AR, ER and VDR assays sporadic activations were observed. Moreover, no activity was observed on the Nrf2 reporter gene assay. Wastewater and street runaway streams from Valencia could be responsible for enhanced activities in one of the water inputs in the Natural Park 'L'Albufera'.

Conclusions: Water quality of relevant wetlands from the Spanish Mediterranean coastline has been evaluated. The utilization of a panel of 5 different bioassays to cover for different toxicity endpoints has demonstrated to be a good tool to assess water quality.

1. Introduction

The International Convention on Wetlands estimates that surface water bodies such as lakes, rivers, marshlands, estuaries and aquifers serve as natural reservoir for the majority of available freshwater worldwide. However, that only represents < 1% of the overall amount of water in the aquatic system [1]. On 2010, the General Assembly of the United Nations recognized the access to freshwater as a human right [2] although more than 2200 million people do not yet have access to salubrious freshwater to cover their necessities [1]. Additionally, the World Health Organization establishes access to safe freshwater as indispensable for health [3]. Consequently, substantial efforts should be made to ensure water quality of natural freshwater reservoirs.

Over the recent decades, chemical analysis has been widely used for the assessment of water quality and the detection of new chemicals of emerging concern [4–9]. However, wide-scope chemical analyses still have many limitations and it may only cover a small part of the pollutants, mainly related to human activities (industry, agriculture and urbanization), that might potentially be present in water bodies. In addition, no information is provided on potential toxic effects of mixtures of natural and anthropogenic chemicals [10,11]. In this sense, effect-based methodologies can be of help by revealing biological activities that could be relevant to adverse effects in freshwater organisms [11–13]. Thus, the utilization of bioanalytical methodologies to assess water quality can bridge the gap between chemical analysis and real environmental status [10,14]. As a complementary tool to chemical analysis, effect-based methodologies can, therefore, detect toxicity posed by untargeted compounds or chemical mixtures [14]. Several studies have used bioassays to assess toxic activities in surface water bodies [15–20].

Selecting relevant toxicity endpoints based on the nature of the water samples is key for a comprehensive assessment of water quality [21]. The in vitro bioanalytical approach used in this work consisted of a panel of 5 bioassays as markers of relevant toxicity endpoints in surface water samples due to the potential to detect combined effects of organic micropollutants. Aryl

hydrocarbon receptor (AhR) has diverse physiological functions related to chemical and microbial defence, reproduction, development, energy metabolism, immunity and inflammation [22] and AhR activity is often observed in surface water samples based on, *e.g.*, elevated presence of aromatic hydro alkyl substances in the environment. Although this might not be an adverse effect per se, it highlights the presence of bioactive chemicals in the water sample [23,24]. The presence of endocrine disruptive chemicals and hormones can be evaluated by means of androgen receptor (AR) and estrogen receptor (ER) activation or inhibition. The control of such types of chemical species in surface water ecosystems is relevant to prevent from intense distress of the normal physiology of exposed organisms [24,25] due to the relevant role of androgens in the development and function of the immune, musculoskeletal, reproductive, cardiovascular and neural systems, and estrogens being essential for bone strength, cardiovascular function, reproduction, cognitive behaviour and gastrointestinal systems [26,27]. Additionally, oxidative stress response, measured by means of the nuclear factor erythroid 2-related factor 2 (Nrf2) activity, is a good indicator of environmental pollution [23,24,28] since this receptor is often triggered by the presence of organic micropollutants in aquatic samples. Oxidative stress can result in different adverse outcomes such as carcinogenicity, tissue damage or teratogenicity [29,30]. The cellular defence mechanism against oxidative stress, regulated by Nrf2, can be used to assess the presence of oxidative stress inducing compounds in water samples. Finally, vitamin D endocrine signalling, transduced via the vitamin D receptor (VDR), plays important roles in multiple physiological systems. A disruption of this signalling pathway would be defined as an endocrine disrupting effect, which could potentially have serious effects to both humans and the ecosystem [31].

In this work, we aimed to assess the toxic activities of different water bodies along the Spanish Mediterranean coastline. In this region, water scarcity has become of major concern. It is expected that climate change will exacerbate this phenomenon; therefore, it is essential to evaluate water quality of significant natural reservoirs. Selection of wetlands was based on the list of Wetlands of International Importance [32] along with other locally relevant water bodies.

Among the sampled locations, Ebro River and Albufera Natural Park (Valencia, Spain) are of special interest as they might be affected by effluent wastewater streams of big cities and industries. This work reflects the first comprehensive study covering a broad set of samples from Mediterranean Spanish coastline and evaluating their water quality by using a set of 5 bioassays for 8 different toxicity endpoints.

2. Materials and methods

2.1. Collection of samples

In this study, 11 water samples were collected from relevant water bodies along the Spanish Mediterranean coastline (**Figure 1**). The sampled locations, including both river water and coastal lagoon water, were selected based on regional significance and/or their inclusion in the list of Wetlands of International Importance (8 out of 11 samples) [32]. **Table 1** summarizes the relevance of the sample as well as the potential impact of surrounding activity. Briefly, samples CL2 and CL7 were collected in areas surrounded by rice agriculture, even CL2 having instream water from irrigation of rice camps. Samples CL5, CL6, CL10 and CL11 were collected in areas with a prominent level of citrus-fruit agriculture with the associated likelihood of impact by pesticides in those samples. Otherwise, samples CL3, CL4 and CL9 were collected in points where human activity can be of importance (CL3 and CL4 close to urbanized areas, and CL9 within a highly touristic part of 'Albufera' natural park). Finally, sampling points for CL1 and CL8 might be affected by treated wastewater streams and urban runaways from large cities, although CL1 is a flowing river that might reduce its impact into water quality.

Grab samples (8 L) were collected in polyethylene terephthalate (PET) plastic bottles in February 2019. Prior to sample collection, bottles were rinsed three times with the water sample. After collection, water samples were stored at 4 °C until extraction performed within 24 h. Alongside wetland water samples, two procedural blank samples were also collected under the same conditions. Milli-Q water (8 L) were collected in PET bottles and stored at 4 °C until extraction to account for potential migration of chemicals from sampling containers into the aqueous phase.

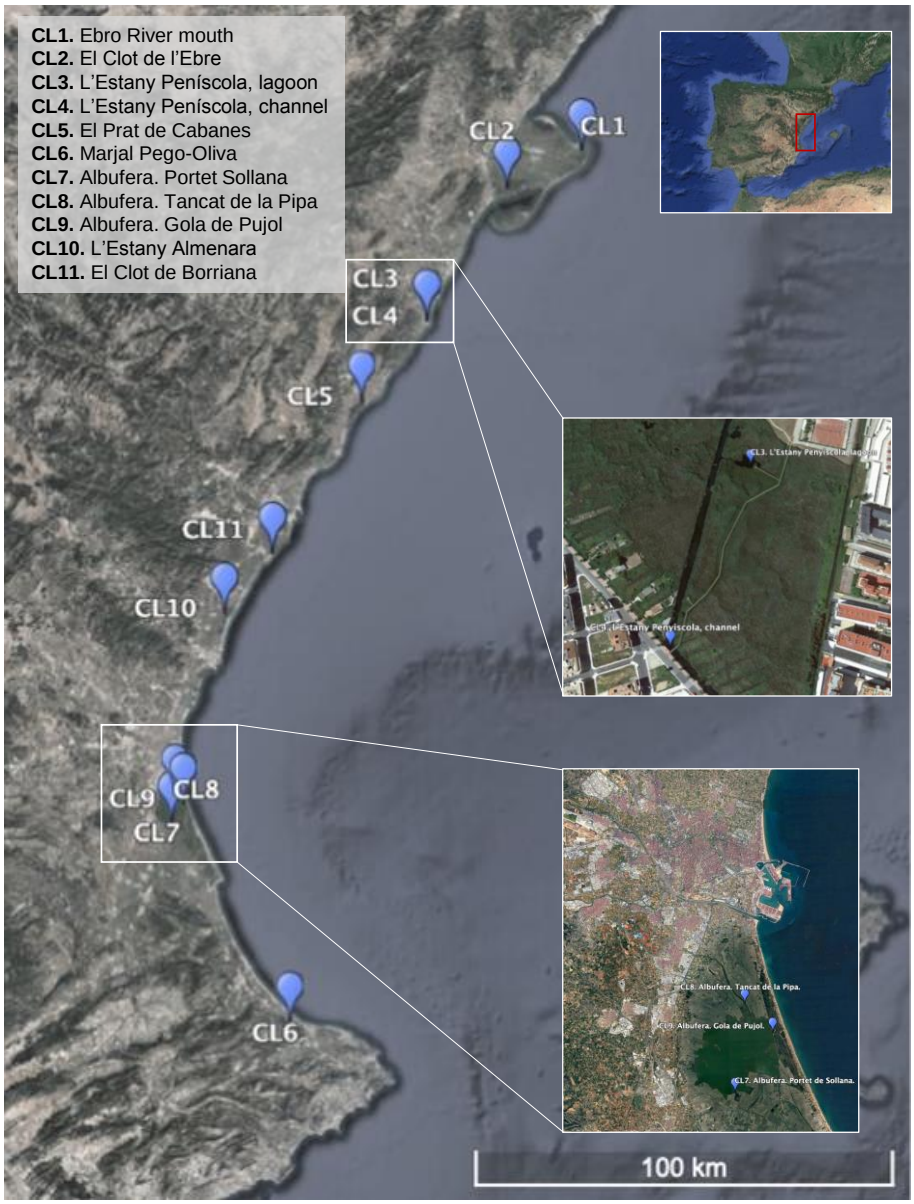

Figure 1. Sampling points across Spanish Mediterranean coastline. Magnified areas show locations where sampling points were nearby. Upper-right map indicates the region within the Iberian peninsula under study (red square).

Table 1. Sample location details, relevance of sample and observations.

ID	Sampling location	Coordinates	Relevance	Observations
CL1	Ebro river mouth	40° 43' 10.20" N 00° 51' 20.88" E	Part of 'Delta del Ebro' natural park. Included in list of Wetlands of International Importance [32].	Largest river in Spain. Instream of treated wastewater from large populations.
CL2	El Clot de l'Ebre	40° 38' 35.52" N 00° 38' 24.36" E		Instream of irrigation water from rice agriculture
CL3	L'Estany Peníscola, lagoon	40° 21' 59.04" N 00° 24' 01.80" E	Flora micro reserve. Designated as Place of Community Interest by regional government.	Potentially affected by human activity.
CL4	L'Estany Peníscola, channel	40° 21' 51.12" N 00° 23' 56.76" E		
CL5	Prat de Cabanes-Torreblanca	40° 11' 50.28" N 00° 12' 31.32" E	Included in list of Wetlands of International Importance [32].	Area with potential impact of citrus-fruit agriculture.
CL6	Marjal Pego-Oliva	38° 52' 23.52" N 00° 02' 53.88" W	Included in list of Wetlands of International Importance [32].	Area with potential impact of citrus-fruit agriculture.
CL7	Albufera. Portet Sollana	39° 18' 37.08" N 00° 21' 25.92" W	Part of 'L'Albufera' natural park. Included in list of Wetlands of International Importance [32].	Area with potential impact of rice agriculture.
CL8	Albufera. Tancat de la Pipa	39° 22' 05.52" N 00° 20' 45.60" W		Instream of treated wastewater and urban runaways from the city of Valencia (~2,500,000 inh., Spain)
CL9	Albufera. Gola de Pujol	39° 20' 14.04" N 00° 11' 32.64" W		Highly tourist area of the natural park. Impact of human activity.
CL10	L'Estany Almenara	39° 45' 14.04" N 00° 11' 32.64" W	Included in list of Wetlands of International Importance [32].	Area with potential impact of citrus-fruit agriculture.
CL11	El Clot de Borriana	39° 52' 46.92" N 00° 03' 14.04" W	Natural park supplied with water from a natural spring.	Area with potential impact of citrus-fruit agriculture.

2.2. Sample Treatment

Water samples (2.5 L) were extracted by means of offline Solid Phase Extraction (SPE) using Oasis HLB cartridges (20 cc, 1 g) from Waters Corporation (Milford, MA, USA). Cartridges were previously conditioned with 3 × 10 mL of methanol

(LC–MS grade, Scharlab, Barcelona, Spain) and 2 × 10 mL Milli-Q water (Millipore, Bedford, MA, USA). In order to avoid clogging, silanized glass wool was located inside the cartridge so bigger particulate matter could be retained and easily discarded. After sample loading, SPE cartridges were eluted with 3 × 10 mL of methanol. The three eluates were pooled and evaporated at 40 °C under N_2 beam (0.7 bar) using a TurboVap II system (Caliper LifeSciences, Hopkinton, MS, USA) for 45 min reaching a final volume of 0.2 mL. Ethanol (absolute grade, Scharlab, Barcelona, Spain) was then added up to a final volume of 1 mL and extracts were frozen overnight at − 20 °C. Finally, extracts were centrifuged at 12,000 rpm for 10 min to remove fine particulate matter. A final concentration factor of 2500 was accomplished. Sample extracts were stored at − 20 °C until analysis.

Procedural blank samples, consisting of Milli-Q water, underwent the same sample treatment as wetland water sample to account for potential impact of sample treatment on the bioactivity of samples.

2.3. Bioanalysis of surface water samples

Wetland water samples, procedural blanks and positive controls were evaluated for AhR, agonistic AR (AR+) and antagonistic AR (AR−), agonistic ER (ER+) and antagonistic ER (ER−), Nrf2, and agonistic VDR (VDR+) and antagonistic VDR (VDR−) activities in reporter gene assays (**Table 2**). Cytotoxicity was evaluated in all cell lines using cell viability assays (MTS-based colorimetric assay and CellTiter-Glo® luminescent cell viability assay).

The activity of AhR was evaluated in transiently transfected human hepatocarcinoma cells (HepG2), which were transfected with a luciferase reporter plasmid under control of a DNA element responsive to ligand activated AhR (donated by Prof. Michael Denison, University of California, Davis, USA) [33]. AR+ and AR− activity was studied in the stably transfected Chinese Hamster Ovary cell line AR-EcoScreen™ (National Institutes of Biomedical Innovation, Health and Nutrition JCRB cell bank) with a human AR expression construct and a luciferase reporter construct under the control of the androgen response element [33]. ER+ and ER− activities were evaluated in a variant of human breast

carcinoma MCF7 cell line, VM7Luc4E2 (donated by Prof. Michael Denison, University of California, Davis, USA), which contains a stably integrated ER-responsive luciferase reporter plasmid [33]. AR and ER activities were analyzed mainly according to OECD guidelines [33,34]. The stably transfected human breast adenocarcinoma cell line MCF7C32ARE was used to measure oxidative stress corresponding to Nrf2 activity, and was kindly provided from R. Wolf (University of Dundee, Nethergate, Scotland) [35]. Finally, VDR+ and VDR− activity were evaluated in human embryonic kidney cell line HEK 293, containing a human vitamin D receptor ligand-binding domain and a Gal4 DNA binding domain as well as beta-lactamase reporter gene under the control of a UAS response element (VDR-UAS-bla HEK 293T) (ThermoFisher Scientific, Stockholm, Sweden). Further details and an expanded description of activity and cell viability assays are available in **Section S1** of **Supporting Information**.

Table 2. Panel of reporter gene assays for the assessment of toxicological fingerprint of wetland samples. Cell lines used, cytotoxicity assay method and positive control used for bioanalysis.

Bioassay		**Cell line**	**Cytotoxicity assay**	**Bioactivity positive control**
Aryl hydrocarbon receptor		Human hepatocellular carcinoma (HepG2)	MTS-based colorimetric assay	2,3,7,8-Tetrachlorodibenzodioxin (TCDD)
Androgen receptor	AR +	Chinese Hamster Ovary (AR-EcoScreen)	MTS-based colorimetric assay	Dihydrotestosterone (DHT)
	AR -			Hydroxyflutamide (OHF)
Estrogen receptor	ER +	VM7Luc4E2	CellTiter-Glo® Luminescent Cell Viability Assay	17β-estradiol (E2)
	ER -			Raloxifene (Ral)
Nuclear factor erythroid 2-related factor 2		MCF7C32ARE	MTS-based colorimetric assay	Tert-butylhydroquinone (tBHQ)
Vitamin D receptor	VDR +	VDR-UAS-bla HEK 293T	MTS-based colorimetric assay	1α, 25-dihydroxyvitamin D3 (1,25-D3)
	VDR -			*n.a.*

Positive controls for each bioassay (**Table 2**) were analyzed alongside wetland water samples, procedural blanks and vehicle controls. For antagonistic effects, cells were co-treated with an agonistic stimulator as negative control test at a concentration corresponding to approximately EC_{80}. 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) and tert-butylhydroquinone (tBHQ) were used as positive control for AhR and Nrf2 reporter gene assays, respectively. In ER reporter gene assay, 17β-estradiol (E2) was used as control for agonistic activity and raloxifene (Ral) for antagonistic activity. For the AR bioassay, dihydrotestosterone (DHT) was used as a positive control for agonistic activity and hydroxyflutamide (OHF) for antagonistic. Finally, 1α, 25-dihydroxyvitamin D3 (1,25-D3) was used as a positive control for VDR+ activity. No appropriate positive control for VDR− activity is commercially available and, as a consequence, it could not be controlled. The positive controls in the reporter gene assay were analyzed in 6–12 concentration levels to obtain a standard calibration curve.

For incubation with cells, wetland water sample and procedural blank SPE extracts (2500 times enriched) were diluted 100 times with cell medium to get a final concentration of 1% ethanol. In consequence, the relative enrichment factor (REF) in the bioassays was 25. All water samples were tested for cell viability and bioactivity in concentration–response relationships (REF = 25, 12.5, 6.75 and 3.125) with 4 replicates for each concentration as previously proposed by Mehinto *et al.* [36]. In all experiments, vehicle controls were included, consisting of 1% ethanol, equivalent to water sample ethanol content. Vehicle controls were tested in 8 replicates.

2.4. Data processing

Bioactivities of wetland water samples, procedural blanks and positive controls we normalized to vehicle controls on each plate. Bioactivity was then expressed as fold-change compared to VHC (set as 1). Standard curves for AhR, AR, ER and VDR (nuclear receptor bioassays) were drawn by fitting data to a four-parameter sigmoidal curve. For Nrf2, since no maximum effect can be reached, standard data were fitted to a liner regression. No appropriate standard is available for

the antagonistic VDR reporter gene assay, and therefore, no standard curve could be obtained.

Cut-off values for cytotoxicity were set at 0.8 compared to VHC set at 1. Cut-off values for bioassays, *i.e.*, for classification of samples as active, were based on the limit of detection (LOD) calculated as 1 plus 3 times the standard deviation of the VHC from all plates within the experiment for agonistic assays; and 1 minus 3 times the standard deviation of the VHC from all plates within the experiment for antagonistic assays [37]. Cut-off values for AR antagonistic assays were established following OECD guideline [34]. Wetland samples were analyzed at different concentrations to enable the calculation of effect concentration (EC) values by means of statistical analysis. Given the observed activity for each sample at REF = 1, the bioanalytical equivalent concentration (BEQ) was interpolated from the dose–response curve for the positive control. For this purpose, concentration–response curve of each sample was adjusted to a linear regression [37]. Statistical analysis and graphical presentation were performed using GraphPad Prism 6.01.

3. Results and discussion

3.1. Cell viability

Cell viability was evaluated in all cell lines used in the study for the whole set of VHC, procedural blanks and wetland samples at different concentrations (**Figure S1** of **Supporting Information**). In general, no cytotoxicity was observed for any sample in any cell line except for sample CL8. The highest concentration tested (REF = 25) showed a slight cytotoxic effect on VM7luc4E2 cell line (ER assay, **Figure S1b** of **Supporting Information**), MCF7C32ARE cell line (Nrf2 assay, **Figure S1d** of **Supporting Information**) and AR-EcoScreen cell line (AR assay, **Figure S1e** of **Supporting Information**). Attending to these results, it was decided to use the 4 concentrations tested (REFs 25, 12.5, 6.3 and 3.1) to assess the bioactivity of wetland samples in all cell lines, paying special attention to results obtained for CL8 at the highest REF value tested. Procedural blanks and VHC did not show cytotoxicity in any cell line.

3.2. Evaluation of toxicity end-points

The bioactivities of the 11 wetland water samples were evaluated by a panel of 5 reporter gene assays for 8 different toxicity endpoints. **Table 3** shows the bioactivity observed in the samples, the EC_{10} (or IC_{75}) values and the BEQ of the samples with determined EC_{10} (or IC_{75}) values. EC_{10} (or IC_{75}) values can be used for comparison purposes since they indicate the REF required to reach the 10% of the maximum activity observed for the positive control (or the 25% inhibition activity for antagonistic assays). A lower EC_{10} (or IC_{75}) value indicates a more potent activity. Accordingly, values equal to or lower than 1 indicate that activity is detected in the collected surface water sample prior to SPE, or at even lower concentrations. A different way to evaluate the bioactivity of the sample is the calculation of the BEQ; based on the activity observed in the sample, it gives an estimation of the concentration of the positive control needed to inflict the same activity in the water sample at REF 1. Thus, higher BEQ values indicate higher activity of the original water sample.

Table 3. Bioactivity observed in wetland water samples for the panel of bioassays used in the study

		Cut-off (fold-change)	EC / BEQ	Wetland samples										
				1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
AhR		2.0	EC_{10}	24.7	>25	>25	>25	19.5	10.6	13.3	7.9	21.6	-	-
			TCDD-eq (pM)	10.2	<7.7	<6.9	<9.3	12.4	20.5	17.1	22.2	11.3	-	-
AR	AR+	1.5	EC_{50}	-	-	-	-	-	-	-	-	-	-	-
			DHT-eq (pM)	-	-	-	-	-	-	-	-	-	-	-
	AR-	0.7	IC_{75}	-	-	-	-	-	-	-	*d.*	-	-	-
			OHF-eq (pM)	-	-	-	-	-	-	-	-	-	-	-
ER	ER+	1.5	EC_{50}	-	-	-	-	-	-	-	-	-	-	-
			E2-eq (pM)	-	-	-	-	-	-	-	-	-	-	-
	ER-	0.8	IC_{75}	-	-	-	*d.*	*d.*	*d.*	-	3.21	3.84	*d.*	*d.*
			Ral-eq (nM)	-	-	-	-	-	-	-	441	23.1	-	-
Nrf2		1.5	$EC_{IR1.5}$	-	-	-	-	-	-	-	-	-	-	-
			tBHQ-eq (µM)	-	-	-	-	-	-	-	-	-	-	-
VDR	VDR+	1.5	EC_{50}	-	-	-	-	-	-	-	-	-	-	-
			1,25-D3-eq (pM)	-	-	-	-	-	-	-	-	-	-	-
	VDR-	0.7	IC_{75}	-	-	-	-	-	-	-	*d.*	-	-	-

- : no activity detected above limit of detection at any REF; *d.:* activity detected above limit of detection but no dose-response observed; EC_{10}: sample REF value to produce 10% of the maximum effect produced by positive control; EC_{50}: sample REF value to produce 50% of the maximum effect produced by positive control; IC_{75}: sample REF value to produce 25% of the maximum antagonistic effect produced by positive control; $EC_{IR1.5}$: sample REF value to induce a 1.5 fold-change activity versus vehicle control; BEQ: bioequivalent concentration; TCDD: 2,3,7,8-Tetrachlorodibenzodioxin; DHT: Dihydrotestosterone; OHF: Hydroxyflutamide; E2: 17β-estradiol; Ral: Raloxifene; tBHQ: Tert-butylhydroquinone; 1,25-D3: 1α, 25-dihydroxyvitamin D3.

Among the bioassays analyzed, AhR seems to be most responsive showing activity for all samples except for CL10 and CL11 (**Table 3**). From another perspective, sample CL8 seems to be the most polluted sample showing activity in every reporter gene assay except for Nrf2. This sample was collected in an area potentially affected by wastewater (either treated or untreated) and street runaways from a big city in the Spanish Mediterranean Coast (**Figure 1**). Therefore, it was expected to be the most polluted sample and, as a consequence, the one showing more activity in the assays.

Procedural blanks were analyzed for all toxicity endpoints and no activity was detected for any of them (**Figure S2** of **Supporting Information**).

3.2.1. Aryl hydrocarbon receptor bioactivity

Figure 2 highlights the bioactivity observed for the AhR reporter gene assay for both the positive control and wetland samples. Linear regression was performed on the concentration–response data and used for calculation of the EC_{10} value. Neither of the REF values tested for samples CL10 and CL11 exceeded the cut-off value. Consequently, samples CL10 and CL11 were classified as inactive. However, bioactivity for samples CL1 to CL9 was detected above the cut-off value and the activity increased with increasing REF values. The REF values calculated to achieve the EC_{10} are shown in **Table 3**. As indicated, samples CL2, CL3 and CL4 were the least active samples with EC_{10} values > 25; and samples CL6, CL7 and CL8 were the most active samples for the AhR bioassay. The relatively high bioactivity observed in the surface water bodies studied indicated the presence of bioactive chemicals in the samples. Although this might not be an issue for environmental water samples [23,24], it highlights that these samples can potentially activate the AhR, with multiple physiological functions involving energy metabolism, chemical and microbial defence, reproduction, development, immunity and inflammation [22]. At this moment, it is unknown whether this activity is being caused by anthropogenic chemicals or naturally occurring compounds, therefore the results observed cannot lead to the conclusion that water quality of the studied wetland is inadequate. In this respect, the highest activity observed for sample CL8 might indicate the elevated presence of small chemicals inducing AhR effect. Bioanalytical equivalents (BEQ) at REF 1 were calculated by means of the linear range of the concentration–response curve in the samples as well as the dose–response curve for TCDD. The active samples had BEQ values in the range 7.34–22.24 pM of TCDD-Eq, with CL8 showing the highest BEQ value among samples (**Table 3**).

In general, AhR activity in environmental surface water samples has been reported repeatedly. Other studies involving natural water bodies found all surface water assessed bioactive for AhR [28,38,39]. In addition, Lundqvist *et al.*

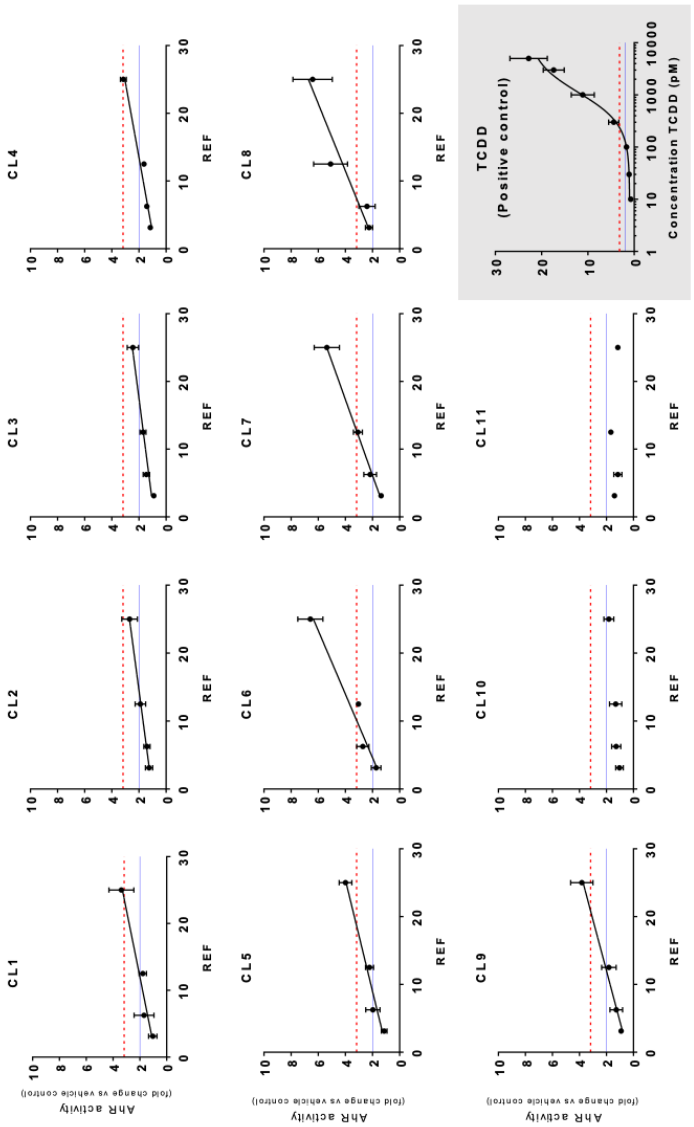

Figure 2. AhR bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 ($n = 4$ at each REF value) and positive control TCDD (grey-shadowed area). Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control), blue line indicates cut-off value for activity value and red dotted line indicates the 10% of assay maximum for comparison purposes.

[40] identified a correlation between the activity observed and the total content of pesticides found in the sample denoting that samples with an increased exposure to agricultural activities may show a larger bioactivity for AhR reporter gene assay. Also, water bodies affected by industrial wastewater discharges results in an enhanced AhR activity [38] as it would be the case for sample CL8.

3.2.2. Androgen receptor (ant)agonistic bioactivity

In general, no bioactivity was observed with the AR reporter gene assay. Individual results for agonistic and antagonistic bioassays are shown in **Figures S3** and **S4** of the **Supporting Information**, respectively. In both analyses, appropriate dose–response activities were observed for the positive controls (DHT for agonistic and OHF for antagonistic).

Although no agonistic activity above the cut-off value was detected in any wetland samples, CL8 showed a decrease in the AR+ activity at higher concentrations. This diminution in the activity observed might respond to the slight cytotoxicity effect observed on the cell line used for the bioassay (**Figure S1** of the **Supporting Information**) at high REF values. Apart from CL8, the other samples analyzed did not show any dose–response trend highlighting no remarkable presence of hormonal activity for the AR+ in the surface water bodies under study.

Similar results were obtained for the AR- reporter gene assay. Only sample CL8 presented activity below cut-off value at REF 25 with a concentration–response curve. However, the apparent increase of antagonistic activity might be due to the cytotoxic effect observed of CL8 at REF 25 in AR-EcoScreen cell line (**Figure S1** of the **Supporting Information**) rather than being antagonistic active. None of the other samples analyzed showed detectable AR–activity. However, an increase over 1 in the activity value can be observed for the vast majority of them although data are normalized to the stimulated vehicle control activity. This behavior can be explained based on a potential cocktail effect between matrix-endogenous chemicals and the DHT added to stimulate the AR. Yet, none of them showed inhibition (except for CL8).

Detection of androgenicity or antiandrogenicity in surface water samples is common. Several studies have detected AR (ant)agonistic activities in wide sampling campaigns [19,28,33,38] while others did not detect bioactivity for the AR reporter gene assays [41].

3.2.3. Estrogen receptor (ant)agonistic bioactivity

Estrogenicity and antiestrogenicity was evaluated by means of an ER-responsive luciferase reporter plasmid stably integrated in a cell line. ER+ activity is reported in **Figure S5** of the **Supporting Information**. As illustrated, appropriate dose–response was observed for E2 (positive control), although no samples showed estrogenicity above the cut-off value. However, CL8 sample showed a clear decreasing trend in the activity fold-change (compared to vehicle control) that can be related to the cytotoxicity observed of such sample at REFs 12.5 and 25. In the case of ER-, the observations are more complex. Several samples showed activity below the cut-off value (CL4, CL5, CL6, CL8, CL9, CL10 and CL11) (**Figure 3**); however, no dose–response was observed for CL4, CL5, CL6, CL10 and CL11 and, therefore, the activity could only be detected (**Table 3**) and no IC or BEQ value could be calculated. On the contrary, CL8 and CL9 showed a clear dose–response trend. Linear regression is usually used for the interpolation of IC_{75} and BEQ values when the activity observed is up to 30% of the positive control; however, the inhibition observed for those samples is around 60 or 70% of that from the positive control and, therefore, sigmoidal fitting is needed to be applied for a better adjust of the curve. Using this approach, CL8 and CL9 has BEQ in the range of 23–441 nM of Ral-eq. It is noteworthy that slight cytotoxicity was observed at high REF values for CL8 (**Figure S1** of the **Supporting Information**) and, therefore, the elevated BEQ observed might be overestimated.

Estrogenic activity is triggered by the presence of natural and synthetic hormones, and to a lower extent by alkylphenols or phytoestrogens [18], whereas, anti-estrogenic activities are induced by dioxin-like substances, polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls [42]. Several studies have detected ER activity at relatively low levels [19,28,33,38,40] with König *et al.* observing strong estrogenic effects in river water samples from Serbia [43].

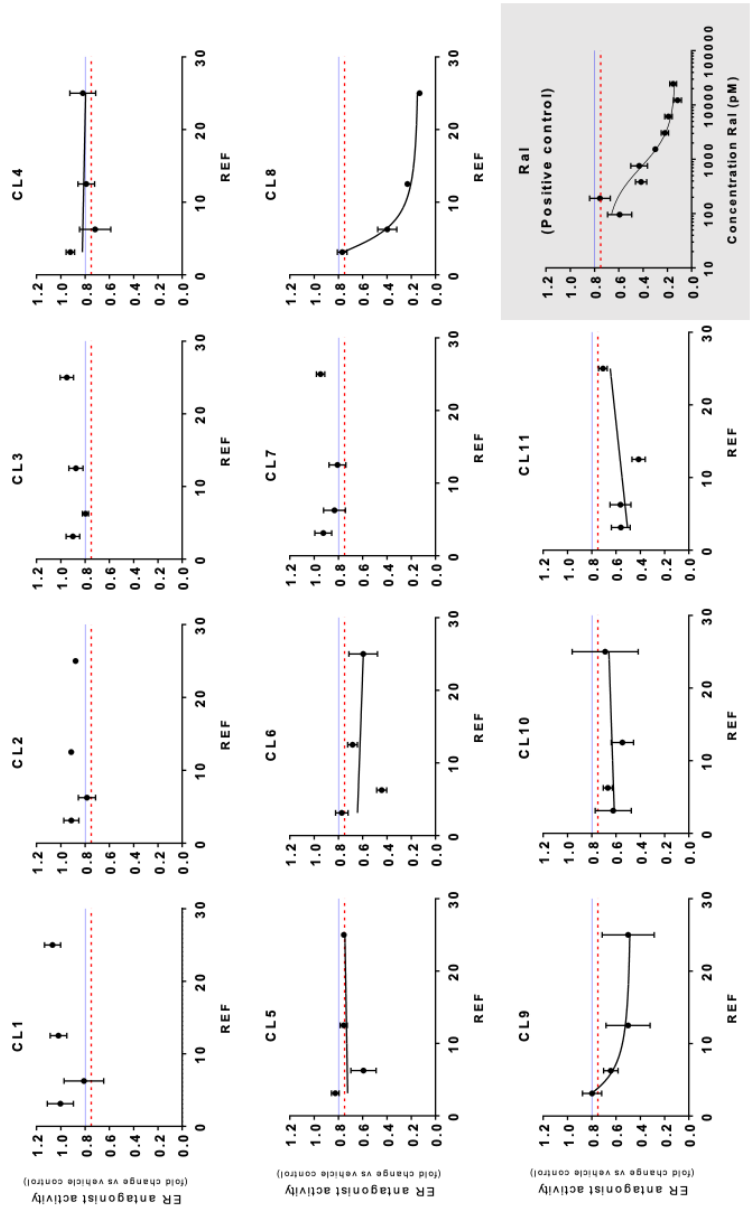

Figure 3. Anti-estrogenicity observed in wetland water samples at REF 3.125, 6.25, 12.5 and 25 ($n = 4$ at each REF value) and positive control Ral (grey-shadowed area). Straight black line indicates the trend curve fitting experimental data, blue line indicates cut-off value and red dotted line indicates the 25% of assay maximum for comparison purposes.

3.2.4. Vitamin D receptor (ant)agonistic bioactivity

The vitamin D endocrine system regulates multiple important physiological functions and is conserved across many species. If this endocrine system is activated or blocked by environmental pollutants, it could be an endocrine disruptive effect with potentially serious consequences. For the VDR+ reporter

gene assay, good dose–response was obtained for the positive control in the agonistic mode (**Figure S6** of the **Supporting Information**). However, there is no commercially available positive control for the VDR- behavior (**Figure S7** of the **Supporting Information**). For the wetland water samples, no agonistic activity was observed above the cut-off value for any sample and only CL8 showed a slight antagonistic activity at REF 25. However, the VDR- activity of CL8 at REF 25 was only marginally below the cut-off value while all other REFs tested were above. As a consequence, the activity was classified only as detected and no IC value was calculated.

The application of VDR reporter gene assay for the assessment of (ant)agonistic activity in surface water samples has been scarcely explored in the literature. Inoue *et al.* [44] evaluated a set of 4 river water samples from Japan for the activity of VDR finding generally low response. Riegraf *et al.* [42], however, found no activity in a set of wastewater samples. The present work is one of the few studies incorporating the VDR bioassay to evaluate the potential activity of endocrine disrupting chemicals in environmental water bodies.

3.2.5. Nuclear factor erythroid 2-related factor 2 bioactivity

Oxidative stress response, evaluated by means of the Nrf2 reporter gene assay, is depicted in **Figure S8** of the **Supporting Information**. Clearly, none of the samples assessed showed activity above the cut-off value at any REF assessed; and, consequently, no dose–response was observed for any sample. These results support the idea that no oxidative inducing compounds are present in the wetland water bodies studied at detectable and active levels. Previous studies also reported none or sporadic detection of oxidative stress response of environmental water samples [28,40]. However, there are also studies depicting sustained oxidative response activity in surface water samples in Sweden [33], in the Danube River Basin [38] or even pronounced response in a sample collected in the same river basin [16]. The fact that previous studies have often reported oxidative stress response for surface water samples highlights the relevance of not having encountered activity over the Nrf2 reporter gene assay in this study.

3.3. Bioanalysis for the assessment of quality of natural water bodies in Spanish Mediterranean coastline

Assessment of water quality and pollution of natural water bodies from the Spanish Mediterranean coastline by means of a panel of 5 reporter gene assays was performed in the present study, where 8 toxicity endpoints were evaluated. Bioactivity was detected in only 20% of the analyses performed on 11 water samples. Among all of them, AhR activity was detected most frequently and found to be a sensitive indicator of the mixture effect of chemicals present in the water samples. The bioactivities observed in this study for AhR were in the range of 7.7–22.2 pM TCDD-eq (2.5–7.4 ng L^{-1}). It is remarkable that the TCDD-eq observed are similar to those previously measured in other studies. Rosenmai *et al.* [33] detected AhR activities in Swedish lakes at TCDD-eq of 4.2–7.8 ng L^{-1}, while Oskarsson *et al.* [28] detected 1.5 ng L^{-1} in river Göta Älv (Sweden). Lower activities (0.15–0.8 ng L^{-1}) were observed in Santa Cruz river (Arizona, US) by Daniels *et al.* [45]. Although the activity observed for AhR bioassay is in line with other studies about surface water quality assessment by bioanalysis, further research towards the analysis of which chemicals, either naturally occurring or anthropogenic, are causing this effect should be conducted.

The most active sample for the set of bioassays studied was CL8. As abovementioned, this sampling location was affected by wastewater effluents and street runaways from a big city (Valencia, 2.5 million inhabitants) and, therefore, bioactivity results could be expected. Most of the samples analyzed did not exhibit detectable activity, which per se denotes good water quality and low levels of chemical contamination. Considering that the water bodies used for the study are of special interest for the regional ecosystem, this finding can be considered of paramount importance.

4. Conclusions

A panel of 8 toxicity endpoints has been evaluated for a set of 11 surface water samples from environmentally relevant water bodies from the Spanish Mediterranean coastline. In general, only 20.5% of the bioassays applied showed detectable activity with the most relevant reporter gene assay being aryl hydrocarbon receptor. AhR activity was detected in water from 9 of the 11 sampling sites. The highest activity was found 'Albufera–Tancat de la Pipa' which may be influenced by contamination through wastewater effluents from Valencia with 2.5 million inhabitants although more research is needed to confirm this hypothesis. The AhR activities observed were in the same range as activities reported in surface water samples in Sweden and US. Antiestrogenic activity was detected in water from 7 of the 11 sampling sites. No estrogenic, androgenic, antiandrogenic, oxidative stress and VDR activities were detected in any of the samples, apart from sporadic, not dose-dependent activities in a few samples. Thus, the water samples only had minor impact on the studied bioactivities denoting the high water quality and low pollution of the water bodies studied.

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Supplementary data

In this part, a section on materials and methods for cell viability and activity assays is included to have supportive information on the written text. Additionally, 8 figures are included. Figure S1. Cytotoxicity evaluation in all cell lines used in the study. Figure S2. Bioactivity responses for vehicle control (VHC), stimulated VHC, procedural blank samples (BS1 and BS2) and positive controls for AhR, Nrf2, VDR+ , VDR−, AR+ , AR-, ER+ and ER−. Figure S3. AR + bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. Figure S4. AR− bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. Figure S5. ER+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. Figure S6. VDR+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. Figure S7. VDR- bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. Figure S8. Nrf2 bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25.

Supplementary data to this article can be found, in the online version, at https://doi.org/10.1186/s12302-021-00510-1 and in this chapter after the section “References”.

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Supplementary data

Section S1. Materials and methods for cell viability and activity assays.

S1.1. Cell culture

The *human hepatocellular carcinoma (HepG2)* cell-line was used for aryl hydrocarbon receptor (AhR) reporter gene assays. HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (with 4.5 g/L glucose) (DMEM) (Lonza, Basel, Switzerland) supplemented with 2 mM L-glutamine (Lonza, Basel, Switzerland), an antibiotic-antimycotic solution with a final concentration of 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 0.25 µg mL^{-1} amphotericin B) (Gibco, Thermo Fisher Scientific), and 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific).

The *Chinese Hamster Ovary (CHO)* cell-line (AR-EcoScreen, purchased from National Institutes of Biomedical Innovation, Health, and Nutrition JCRB cell bank) was used for the AR reporter gene assay. Stably transfected CHO cells were cultured in DMEM F12 (Sigma) medium supplemented with 5% FBS (Gibco), Penicillin/Streptomycin with a final concentration of 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, (Lonza) 2 mM L-glutamine (Lonza, Basel, Switzerland), 100 µg mL^{-1} Hygromycin B (InvivoGen, USA), and 200 µg mL^{-1} Zeocin (Invitrogen, CA, USA). Experimental medium consists of DMEM F12 (Sigma) medium supplemented with 5% dextran-charcoal treated fetal bovine serum (Thermo Scientific), 4 mM L-glutamine (Gibco, Thermofisher Scientific), Penicillin/Streptomycin with a final concentration of 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, (Lonza).

VM7Luc4E2 cells are routinely cultured in RPMI 1640 (Gibco) supplemented with 8% fetal bovine serum (FBS) (Gibco) and 0.9% penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin, Lonza). 0.55 mg/ml Gentamicin (Gentamicin Sulfate, 50mg/ml, Lonza) was used as positive selector. VM7Luc4E2 cells, were grown in experimental medium consisting of DMEM medium (with 4.5 g/L glucose) (Lonza), 4.5% dextran-charcoal treated fetal bovine serum (Thermo Scientific), 2% L-glutamine (Lonza, Basel, Switzerland), 0.9%

penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin, Lonza) and 0.38 mg/ml Gentamicin (Gentamicin Sulfate, 50mg/ml, Lonza) was used as positive selector.

VDR-UAS-bla HEK 293T were purchased from ThermoFisher Scientific (Stockholm, Sweden). This cell line, based on human embryonic kidney cell line HEK 293, contains a human vitamin D receptor (VDR) ligand-binding domain and a Gal4 DNA binding domain as well as a beta-lactamase reporter gene under the control of a UAS response element. Upon ligand binding to the VDR, the expression of the beta-lactamase reporter gene will increase.

Cells were passaged twice a week and maintained between 5% and 95% confluence. The flasks were coated with 1x Matrigel matrix (Gibco, Invitrogen) in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) (Lonza) and incubated for 15 minutes in a humidified incubator. After coating and incubating, the matrigel medium was aspirated and cells were seeded into the flasks. This cell line was routinely cultured in growth medium consisting of DMEM with GlutaMAX™ (Gibco, Invitrogen) supplemented with 10% dialyzed fetal bovine serum (Gibco, Invitrogen), 0.1 mM non-essential amino acids (Gibco, Invitrogen), 25 mM HEPES buffer (Gibco, Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen), and 80 µg/mL respectively of Hygromycin (InvivoGen) and Zeocin (Gibco, Invitrogen) for selection purposes. For agonist and antagonist assay, cells were plated in assay medium with phenol red-free DMEM (Lonza) medium supplemented with 2% charcoal-stripped FBS (Gibco, Invitrogen), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, Invitrogen), 0.1 mM non-essential amino acids (Gibco, Invitrogen) and 1 mM Sodium pyruvate (Gibco, Invitrogen).

MCF7c32ARE cell line was constructed by Wang *et al* (Cancer Res.2006) by stably transfecting the human breast cancer cell line MCF7 with an ARE reporter plasmid.The cell line was kindly provided by the authors. AREc32 cells were grown in growth medium consisting of DMEM with GlutaMAX™ (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen), and 0.8

mg/mL respectively of G418 (InvivoGen) for selection purposes. For experimental purposes, the above culture medium was used but was devoid of G418.

The cells were cultured in incubator with humidified atmosphere at 37˚C containing 95% air and 5% CO_2. Medium was changed every 2-3 days. Trypsin-EDTA (Gibco, Thermofisher Scientific) was used for sub-culturing of cells.

S1.2. Cell treatment

All water samples were tested for cell viability assays and reporter gene assays and were tested for concentration-response relationships with 4 replicates for each treatment. In all experiments, vehicle controls was included, consisting of 1% ethanol, equivalent to water sample ethanol content. Vehicle controls were tested in 8 replicates.

S1.3. Cell viability

The *MTS-based colorimetric assay* (Cell Titer 96® Aqueous One Solution Cell Proliferation Assay) (Promega) was used for HepG2, MCF7c32ARE, VDR-UAS-bla HEK 293T and CHO cells to assess the cell viability. HepG2 cells were seeded, $2*10^4$ cells $well^{-1}$, in transparent 96-well plates (Costar ® Corning Incorporated), and left to incubate for 48 hours, after which medium was changed. After another 24 h, cells were treated with waste water samples for 24 hours. CHO cells were seeded in respective experimental medium with a density of $9*10^3$ cells $well^{-1}$ of CHO, 55800 cells $well^{-1}$of VDR-UAS-bla HEK 293T and 11700 cells $well^{-1}$ of MCF7c32ARE cells 96-well transparent plates, and left to incubate for 24 hours, after which cells were exposed to dose responsive dilutions with samples for 24 hours. At experiment termination, cell viability assay reagent, 20 µl $well^{-1}$, was added and cells were incubated for approximately 15 minutes (HepG2, MCF7c32ARE) and 30 minutes (CHO, VDR-UAS-bla HEK 293T). Absorbance was measured on a Wallac Victor 1420 microplate reader (PerkinElmer, Waltham, MA, USA) at 490 nm. A decrease in viability of ≥ 20% was considered as cytotoxic.

For VM7Luc4E2, the cell viability was analyzed using the *CellTiter-Glo® Luminescent Cell Viability Assay* (Promega). Cells were cultured in experiment

medium with gentamicin for atleast 48 h. 16,000 cells per well were seeded in white clear bottomed 384-well plates, in experiment medium without gentamicin and allowed to grow for 24 h. Cells were treated with samples in a dose responsive dilutions, diluted in experimental media for 24 h. After the exposure, 25µl of CellTiter-Glo luminescent cell viability assay reagent was added and the luminescence was measured in Tecan plate reader (TECAN, Austria GmbH, Austria) and relative effects on cell viability was calculated in relation to the vehicle control. A decrease in viability of ≥ 20% was considered as cytotoxic.

S1.4. Reporter gene assays

AhR activity

The AhR reporter gene assays, were based on transient transfection protocol in HepG2 cells, whereas the remaining reporter gene assays, were based on stably transfected cell lines. Positive standards were included in all experiments.

HepG2 cells were seeded in white clear bottomed 96-well plates (Corning, USA), $2*10^4$ cells $well^{-1}$ and left to incubate for 48 hours after which medium was changed and transfections were performed. Lipofectamine (30 µL $well^{-1}$) (Invitrogen, Thermo Fisher Scientific, USA) was used as transfection reagent. Cells for the AhR reporter gene assay was transfected with a pGL3 based luciferase reporter plasmid (pGudLuc7.5, 90 ng $well^{-1}$) (donated by Professor Michael Denison, University of California, USA) and 30 ng $well^{-1}$ Renilla luciferase plasmid. The transfection mixture was prepared by adding Lipofectamine to 5 µL $well^{-1}$ Opti-MEM (1x) reduced serum medium (Gibco, Thermo Fisher Scientific, USA). After five minutes, the plasmids were added to 5 µL $well^{-1}$ Opti-MEM (1x) reduced serum medium and the two solutions were mixed gently. 10 µL $well^{-1}$ transfection mixture was added and left for 24 hours after which cells were treated with water samples and standards for 24 hours. TCDD (SUPELCO, Sigma-Aldrich, USA) was tested in concentrations of 10-5000 pM in the AhR reporter gene assay. At assay termination, cells were lysed with passive lysis buffer (20 µL $well^{-1}$) (Promega, USA) for 15 minutes. White sticky tape was attached to plate bottom for the AhR reporter gene assay and total lysate was

used for activity measurement. The Dual-Luciferase® Reporter Assay System (Promega, USA) was used to measure Renilla and Firefly luciferase activity according to manufacturer's protocol. Luminescence was measured on a Tecan plate reader (TECAN, Austria GmbH, Austria) with two automatic injection syringes. The injection volume for the Firefly luciferase reagent and Renilla luciferase reagent was 45 µL well^{-1}. Luminescence measurement was conducted over a 5 s period, 2 s after reagent automatic injection with Firefly and then Renilla luciferase reagent addition.

Nrf2, ER &AR- agonist and antagonist activity

All experiments in stably transfected cell lines were conducted in white clear-bottomed 384 well plates (Corning, NY, USA) over a three day period with cell seeding on day 1, cell treatment with water samples and standards on day 2, and luciferase measurement on day 3.

MCF7c32ARE cells were seeded, 5200 cells well^{-1}, in culture medium without G418. Tert-butylhydroquinone (tBHQ) (Sigma Aldrich, USA) was used as a standard in the Nrf2 reporter gene assay and tested in concentrations of 0.78-25 µM.

VM7Luc4E2 cells were seeded, $1.6 \cdot 10^4$ cell well^{-1}, in experimental medium and 4000 cells well^{-1} of CHO cells in experimental medium. The ER and AR reporter gene assay was conducted in agonist and antagonist mode. 17β-estradiol (E2) (Sigma-Aldrich, USA) was used as a standard in the ER reporter gene assay and tested in concentrations of 0.04-367 pM. In the ER anta mode, $9.18 \cdot 10^{-11}$ M of E2 was added together with the water samples and standards with Raloxifene (Raloxifene HCl, Sigma-Aldrich), $2.45 \cdot 10^{-8}$ -9.57 $*10^{-11}$ M. The weak positive control for agonist mode is $9.06 \cdot 10^{-6}$ M of p,p'-Methoxychlor (Sigma-Aldrich, USA) and antagonist mode is $3.36 \cdot 10^{-6}$ M Tamoxifen (Sigma-Aldrich, USA). In the AR anta mode, 500 pM DHT was added together with the water samples and standards. In the AR reporter gene assay, the standard for activation of the receptor was dihydrotestosterone (DHT) (Sigma-Aldrich, USA) tested in concentrations of 0.001-1000 nM, and in the AR antagonism mode the standard

was hydroxyflutamide (OHF) (Sigma-Aldrich, USA) tested in concentrations of 0.0001-10 μM.

At experiment termination, cells for reporter gene assay were lysed with passive lysis buffer (PLB) (Promega), 10μL well^{-1}, for 15 minutes in 384 well plates. Luciferase activity was measured using the Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions. Luminescence was measured on a Tecan plate reader (TECAN, Austria GmbH, Austria) with an automatic injection syringe. The injection volume for the Firefly luciferase reagent was 10 μL well^{-1}. Luminescence measurement was conducted over a 5 s period, 2 s after reagent was automatically injected with Firefly luciferase reagent

VDR agonist and antagonist activity

The VDR agonist and antagonist reporter assay was done by harvesting the non-DA cells from culture of 70-90% confluency. 20,000 cells/well were seeded into 384-well black wall, clear-bottom assay. Cell-free control wells, unstimulated control wells and stimulated control wells were included for each plate. The cells were then exposed to water samples or positive controls, vehicle controls (ethanol) were tested in 8 replicates, 4 replicates for each treatment and positive control. 1α, 25-dihydroxyvitamin D3 (1,25-D3) was used as agonist positive control ranging from 1.11nM - 0.005nM. VDR antagonistic effects of water samples were assessed by stimulating cells with 0.05 nM (1,25-D3). After 24 h of treatment, reporter activity was measured by adding LiveBLAzer™-FRET B/G substrate mixture (Invitrogen). Fluorescence was measured in the blue channel with excitation filter 409/20, emission filter 460/40 nm and in the green channel with excitation filter 409/20, emission filter 530/30 nm.

S1.5. Data analysis

The cell-free control wells was used for background subtraction. The average blue background was subtracted from all blue emission data and average green background from all green emission data. The blue/green emission ratio for each well is calculated and normalized with vehicle control.

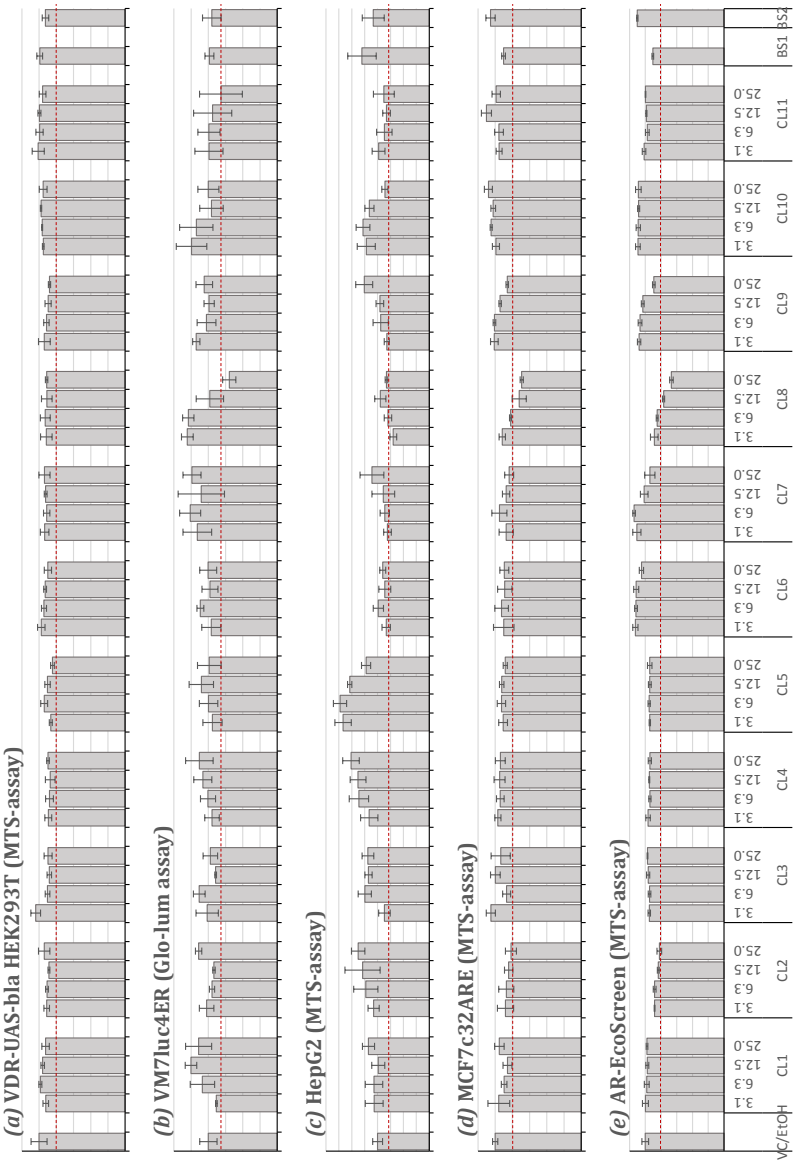

Figure S1. Cytotoxicity assessment of vehicle control (VC), wetland water samples (CL1 to CL11) and procedural blanks (BS1 and BS2). Dotted-red line indicates the cut-off value of 0.8 to declare toxicity.

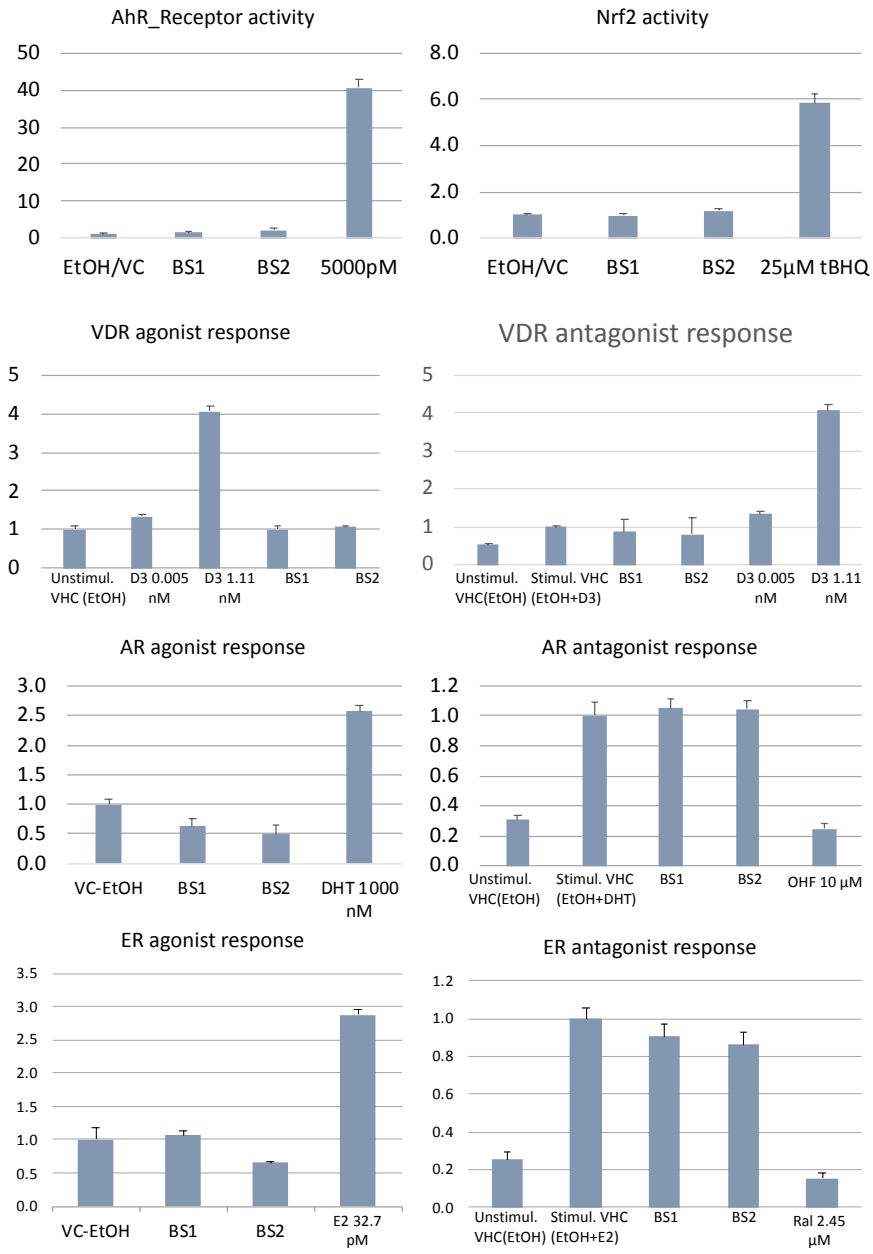

Figure S2. Bioactivity responses for vehicle control (VHC), stimulated VHC, procedural blank samples (BS1 and BS2) and positive controls for AhR, Nrf2, VDR+, VDR-, AR+, AR-, ER+ and ER-.

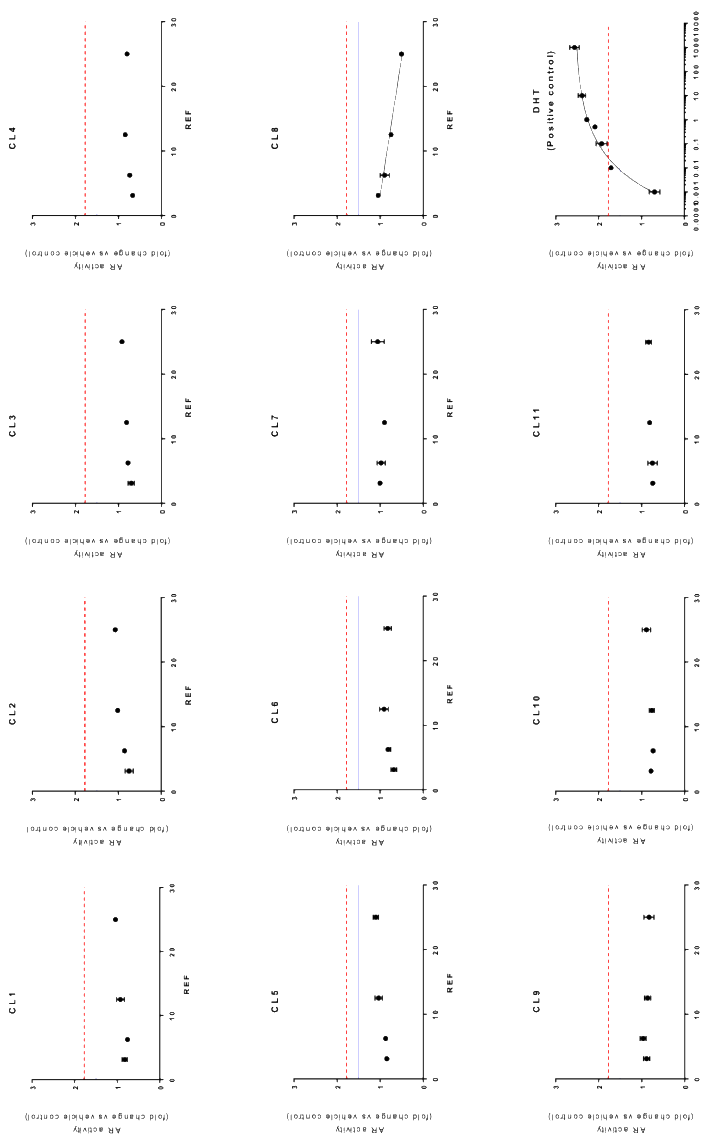

Figure S3. AR+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 (n=4 at each REF value) and positive control DHT. Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control), blue line indicates LOD value and red dotted line indicates the 50% of assay maximum for comparison purposes.

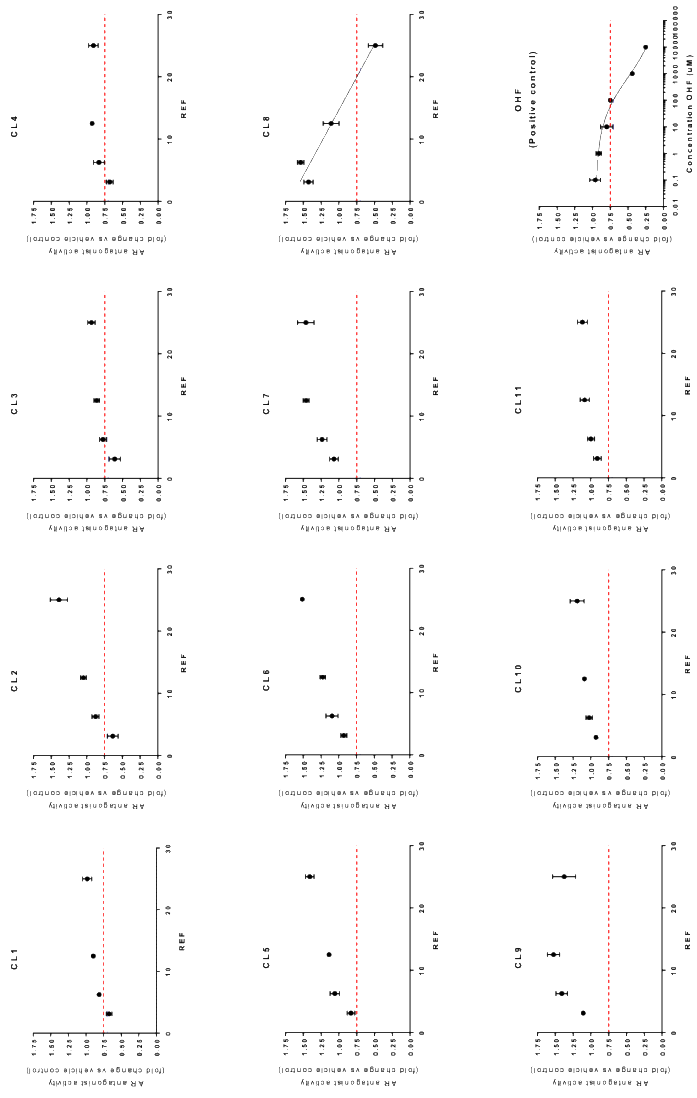

Figure S4. AR- bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 (n=4 at each REF value) and positive control OHF. Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control), blue line indicates LOD value and red dotted line indicates the 25 % of assay maximum for comparison purposes.

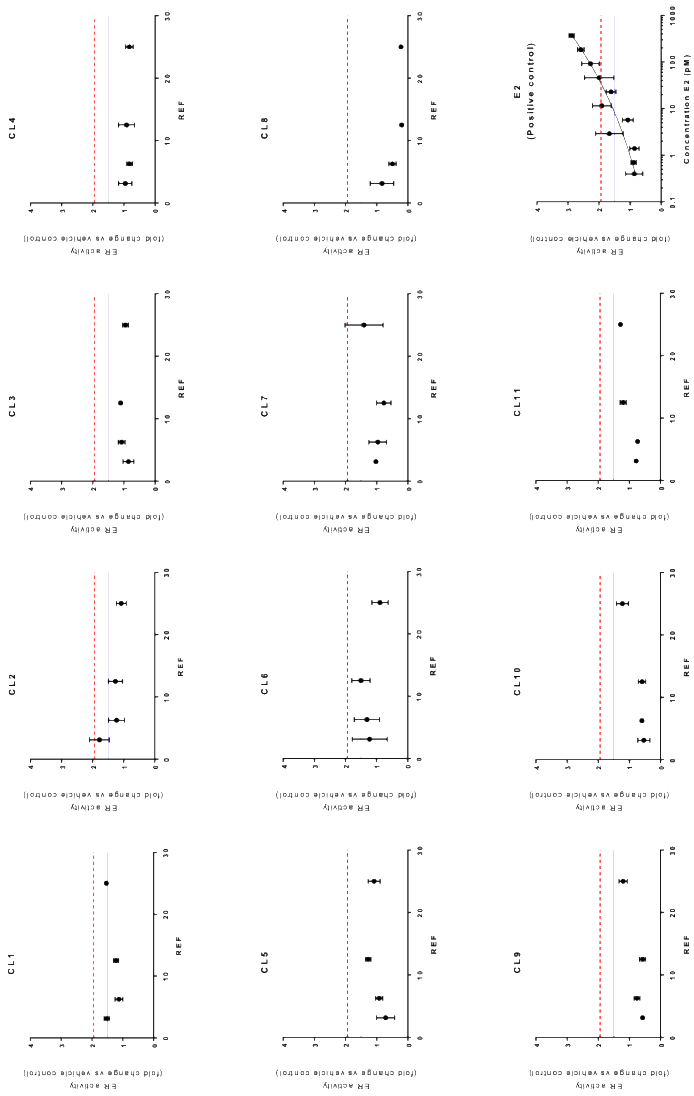

Figure S5. ER+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 (n=4 at each REF value) and positive control E2. Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control), blue line indicates LOD value and red dotted line indicates the 50 % of assay maximum for comparison purposes.

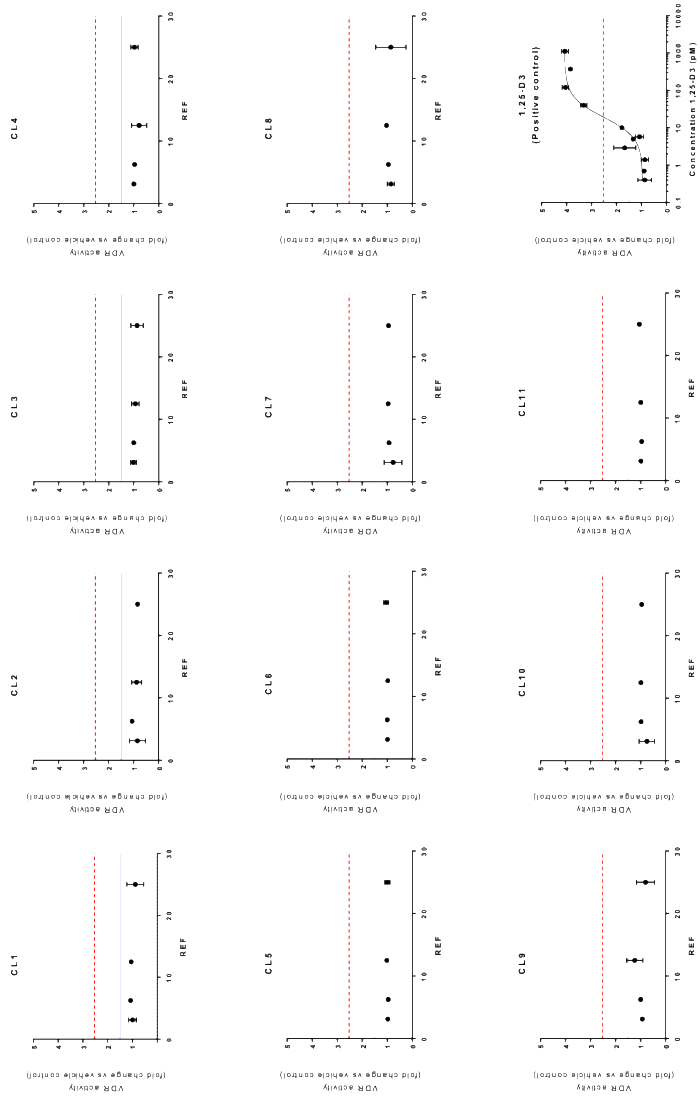

Figure S6. VDR+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 (n=4 at each REF value) and positive control 1,25-D3. Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control), blue line indicates LOD value and red dotted line indicates the 50 % of assay maximum for comparison purposes.

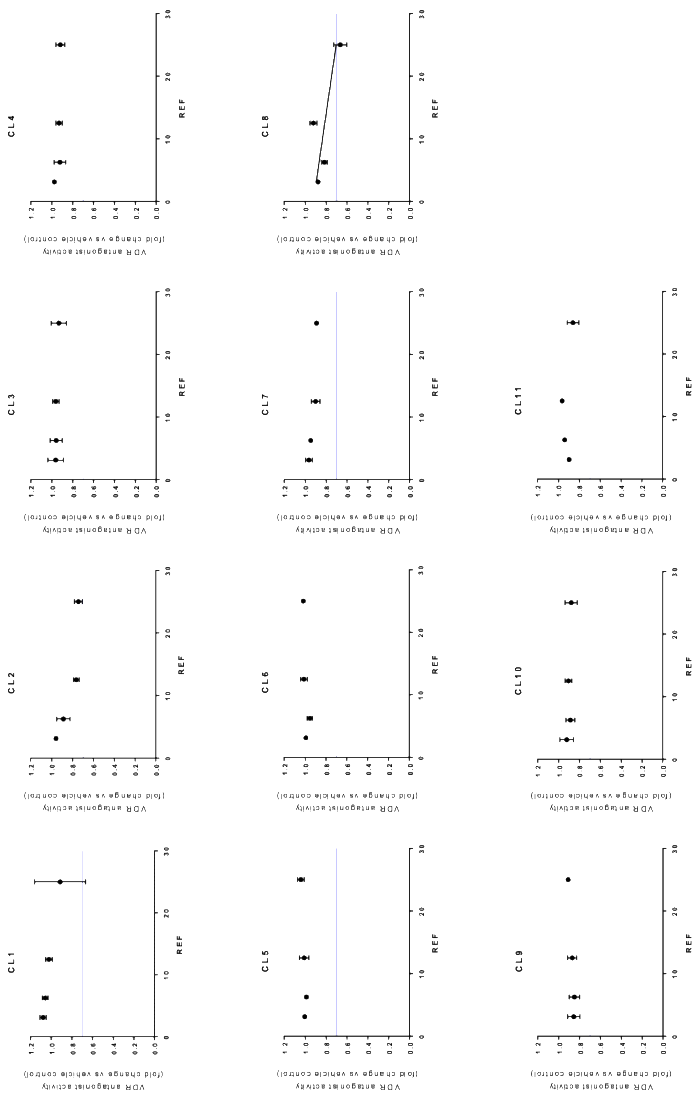

Figure S7. VDR- bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 (n=4 at each REF value). Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control) and blue line indicates LOD value.

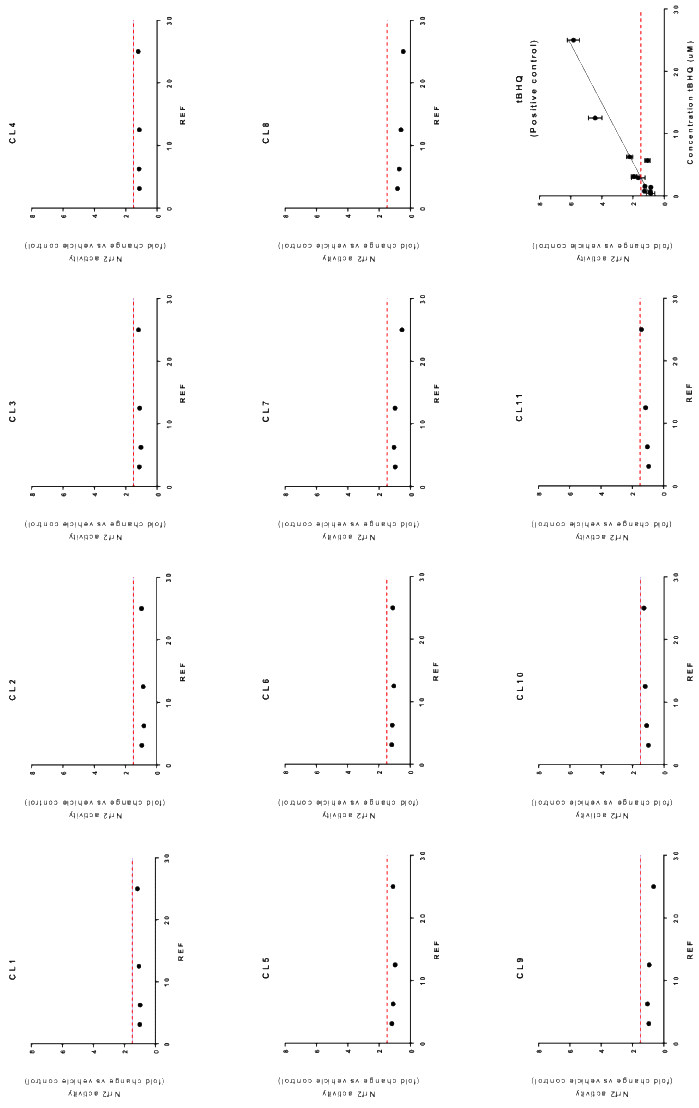

Figure S8. Nrf2 bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 (n=4 at each REF value) and positive control tBHQ. Straight black line indicates the trend curve fitting experimental data (linear regression), blue line indicates LOD value and red dotted line indicates 1.5 fold-change induction of activity of positive control for comparison purposes.

Chapter 4.2.2. Scientific Article 7

INTEGRATION OF CHEMICAL ANALYSIS WITH BIOANALYSIS FOR A COMPREHENSIVE WATER QUALITY EVALUATION IN SPANISH MEDITERRANEAN COASTLINE WATER BODIES

Alberto Celma, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lutz Ahrens and Lubertus Bijlsma

Water Research (2021) Submitted

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Keywords

Ion mobility separation; bioanalysis; Albufera; Ebro river; organic micropollutants screening; water quality.

Abstract

The Spanish Mediterranean basin is particularly susceptible to climate change and human activities, making it vulnerable to the influence of anthropogenic contaminants. Therefore, conducting comprehensive and exhaustive water quality assessment in relevant water bodies of this basin is pivotal. In this work, surface water samples from coastal lagoons or estuaries were collected across the Spanish Mediterranean coastline and subjected to target and suspect screening of hundreds of organic micropollutants by liquid chromatography coupled to ion mobility separation and high resolution mass spectrometry. 91 organic micropollutants could be confirmed and 5 were tentatively identified, being pharmaceuticals and pesticides the most prevalent groups of chemicals. Chemical analysis data was compared with data on bioanalysis of those samples (recurrent aryl hydrocarbon receptor (AhR) activation, and estrogenic receptor (ER) inhibition in wetland samples affected by wastewater streams). The number of identified organic contaminants containing aromatic rings could explain the AhR activation observed. For the ER antagonistic effects, predictions on estrogenic inhibition potency for the detected compounds were used to

explain the activities observed. The integration of chemical analysis with bioanalytical observations allowed a comprehensive overview of the quality of the water bodies under study.

1. Introduction

Climate change, natural resources overexploitation and human activities are affecting the quality of water ecosystems [1]. The Spanish Mediterranean basin has been found to be specially affected by these changes and it is particularly vulnerable to the impact of anthropogenic contaminants [2,3]. A recent study highlighted that the Ebro river (relevant water reservoir in the Northern Spanish Mediterranean area) was affected by elevated concentrations of phosphate originated from agricultural industry reducing the sustainability of the water ecosystem [4]. Similarly, Albufera Natural Park, a typical Mediterranean shallow coastal lagoon located in Valencia, has been affected by many years of negligent management of wastewater streams and a large growing population of surrounding settlements [5]. As a consequence, hundreds of organic micropollutants have been consistently detected in these water bodies [6–10]. Therefore, appropriate strategies for the monitoring of water quality should be applied on these and other vulnerable water ecosystems.

Efforts have been conducted towards the development, application and standardization of comprehensive analytical strategies to monitor the presence of large lists of organic micropollutants [11–16]. The utilization of orthogonal analytical techniques such as ion mobility separation coupled to high resolution mass spectrometry (IMS-HRMS) has allowed the improved identification of small organic molecules in complex matrices [17–19]. Also, the combined application of complementary techniques, such as liquid and gas chromatography coupled to low and high resolution mass spectrometry, has allowed to increase the chemical space and, therefore, the number of contaminants monitored, from non-polar, volatile compounds to (highly) polar, non-volatile compounds, in wide-scope screening strategies [12–14,20–22]. More recently, effect-based methodologies have been applied for a wider and more comprehensive quality evaluation of water samples [23–28]. In such approaches, the combination of chemical and bioanalytical analyses permits the measurement of mixed toxicological (cocktail) effects of compounds as well as to potentially relate them with exposure levels of detected organic micropollutants [24,28,29]. Therefore, by selecting relevant toxicity endpoints

based on the nature of the water samples, effect-based methodologies can give insight into other dimensions of the quality of water samples under study [29].

In the present work, a comprehensive strategy combining ultimate analytical and toxicological tools has been applied for the screening of organic micropollutants of a set of 11 relevant and vulnerable water bodies from the Spanish Mediterranean coastline. To this purpose, a large qualitative target screening was performed for 682 micropollutants using both, ultra-high performance liquid chromatography (UHPLC) coupled to IMS-HRMS (556 compounds) and by UHPLC coupled to low resolution mass spectrometry with triple quadrupole analyzer (UHPLC-MS/MS QqQ) (183 compounds). 57 compounds were monitored by both instrumental configurations. In addition, a complementary suspect screening of more than 900 substances was applied based on UHPLC-IMS-HRMS. Additionally, a large bioanalytical data set was previously obtained from the same samples over different toxicological endpoints to get a comprehensive overview of the quality of the water bodies under study [30]. Briefly, in vitro biological analysis of the wetland samples was performed by means of a panel of 8 different toxicity endpoints including aryl hydrocarbon receptor (AhR), (ant)agonistic androgenicity (AR), (ant)agonistic estrogenicity (ER), oxidative stress response (Nrf2) and (ant)agonistic vitamin D receptor (VDR) reporter gene assays. In this work, we aim to integrate the results from the chemical analyses with the bioanalytical observations to better understand water quality of relevant water bodies from the Mediterranean Spanish coastline.

2. Materials and methods

2.1. Sampling and sample treatment

2.1.1. Sampling locations

In total, 11 water samples were collected from water bodies along the Spanish Mediterranean coastline including both estuaries and coastal lagoon water. Sampling locations were selected based on their inclusion in the list of Wetlands of International Importance (8 out of 11 sites) [31] and/or due to their large diversity of aquatic organisms and wildlife. **Table 1** depicts the relevance of the samples collected as well as the potential impact of specific activities (*viz.* rice agriculture, citrus-fruit agriculture, touristic activity and/or impact of treated wastewater streams and urban runaways from large cities). Evaluating water quality in these Mediterranean wetlands is a major concern, because of the wide variety of contaminants reported in previous studies [6–10], the estimated water loss of 50% over the last century [32], and the dramatic climate changes that might occur in the next years [33–35].

Sampling was performed as follows: grab surface water samples (8 L) were collected in polyethylene terephthalate (PET) plastic bottles previously rinsed with surface water from the sampling location. Samples were collected in February 2019. After collection, water samples were stored at 4 °C until extraction, which was performed within 24 hours. Two procedural blank samples consisting of Milli-Q water were also prepared under the same conditions.

Table 1. Sample collection details, relevance and observations.

	Sampling location	Relevance	Observations
#1	Ebro river estuary (40° 43' 10.20" N; 00° 51' 20.88" E)	Part of 'Delta del Ebro' natural park which is included in list of Wetlands of International Importance [31].	Mouth of the largest river in Spain. Upstream infliction of treated urban wastewater.
#2	El Clot de l'Ebre (40° 38' 35.52" N; 00° 38' 24.36" E)		Rice agriculture irrigation water instream.
#3	L'Estany Peníscola, lagoon (40° 21' 59.04" N; 00° 24' 01.80" E)	Designated as Place of Community Interest by regional government because of the flora and fauna diversity.	Highly tourist area. Potential impact of human activity.
#4	L'Estany Peníscola, canal (40° 21' 51.12" N; 00° 23' 56.76" E)		
#5	Prat de Cabanes-Torreblanca (40° 11' 50.28" N; 00° 12' 31.32" E)	Included in list of Wetlands of International Importance [31].	Potential impact of citrus-fruit agriculture.
#6	Marjal Pego-Oliva (38° 52' 23.52" N; 00° 02' 53.88" W)	Included in list of Wetlands of International Importance [31].	Potential impact of citrus-fruit agriculture.
#7	Albufera. Portet Sollana (39° 18' 37.08" N; 00° 21' 25.92" W)	Part of 'L'Albufera' natural park. Included in list of Wetlands of International Importance [31].	Potential impact of rice agriculture.
#8	Albufera. Tancat de la Pipa (39° 22' 05.52" N; 00° 20' 45.60" W)		Instream of treated wastewater and urban runaways from the city of Valencia (~2,500,000 inh., Spain)
#9	Albufera. Golf de Pujol (39° 20' 14.04" N; 00° 11' 32.64" W)		Highly tourist area of the natural park. Potential impact of human activity.
#10	L'Estany Almenara (39° 45' 14.04" N; 00° 11' 32.64" W)	Included in list of Wetlands of International Importance [31].	Potential impact of citrus-fruit agriculture.
#11	El Clot de Borriana (39° 52' 46.92" N; 00° 03' 14.04" W)	Natural park supplied with water from a natural spring.	Potential impact of citrus-fruit agriculture.

2.1.2. Sample extraction procedure

The extraction procedure was adapted from Celma *et al*. [30]. In brief, 2.5 L of water sample was extracted by solid phase extraction (SPE) using Oasis HLB cartridges (20 cc, 1 g, 60 µm) (Waters Corporation, Milford, MA, USA). Cartridges were previously conditioned with 3 x 10 mL of methanol (LC-MS grade, Scharlab, Barcelona, Spain) and 2 x 10 mL Milli-Q water (Millipore, Bedford, MA, USA). During sample loading, silanized glass wool was deployed inside the cartridge to avoid clogging of stationary phase. After sample loading, SPE cartridges were dried under vacuum and then eluted with 3 x 10 mL of methanol. The three eluates were pooled and evaporated at 40 °C under nitrogen (N_2) beam (0.7 bar) using a TurboVap II system (Caliper LifeSciences, Hopkinton, MS, USA) until reaching a final volume of 0.2 mL. Milli-Q water (Millipore, Bedford, MA, USA) was then added up to a final volume of 1 mL (final concentration factor was x2500 in water:methanol 80:20). Sample extracts were then frozen overnight at -20 °C. The following day, extracts were centrifuged at 12,000 rpm for 10 min to remove precipitates and particulate matter. Finally, sample extracts were stored at -20 °C until analysis.

Prior to extraction, raw water samples as well as procedural blanks were spiked with 15 isotopically labelled internal standards (ILIS) for quality control purposes.

2.2. Chemical analysis

2.2.1. Instrumentation

A large screening of organic micropollutants was conducted over the set of 11 water samples from relevant water bodies of the Spanish Mediterranean coastline and the procedural blanks. Compounds under investigation included >1,500 pharmaceuticals and metabolites, pesticides and transformation products, hormones, mycotoxins and psychoactive drugs.

Samples were screened by UHPLC-IMS-HRMS for 556 target compounds as well as approximately 900 suspect compounds including pesticides and transformation products, pharmaceuticals and metabolites, hormones, drugs

and mycotoxins. Additionally, a screening by liquid chromatography-tandem mass spectrometry with triple quadrupole (UHPLC-MS/MS QqQ) was also applied for 183 target chemicals (hormones, per- and polyfluoroalkyl substances (PFASs) and other organic micropollutants). 57 compounds out of the 183 targets were also included in the UHPLC-IMS-HRMS screening methodology.

2.2.1.1. UHPLC-IMS-HRMS

A Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA) connected to a VION IMS-QTOF mass spectrometer, using electrospray ionization (ESI) interface operating in both positive and negative ionization mode was used for sample analysis. Compounds were separated using a CORTECS® C18 2.1 x 100 mm, 2.7 µm fused core column (Waters) at a flow rate of 300 µL min^{-1} with a gradient consisting of Milli-Q water from Millipore and methanol as mobile phases, both with 0.01% formic acid. Total run time was 18 min. MS data were acquired using the VION in HDMS^e mode, over the range *m/z* 50-1000, with N_2 as the drift gas, an IMS wave velocity of 250 m s^{-1} and wave height ramp of 20-50 V. Leucine enkephalin (*m/z* 556.27658 and *m/z* 554.26202) was used for mass correction in positive and negative ionization modes, respectively. Two independent scans with different collision energies, 6 eV for low energy (LE) and a ramp of 28-56 eV for high energy (HE), were acquired during the run with a scan time of 0.3 s was set in both LE and HE functions. Nitrogen (≥ 99.999%) was used as collision-induced dissociation (CID) gas. Data were examined using an in-house built accurate mass screening workflow within the UNIFI platform (version 1.9.4) from Waters Corporation.

Compounds included in the IMS-HRMS target database can be found at the freely available online repository Zenodo [36] as well as the Suspect List Exchange platform from the NORMAN network [37]. A detailed description of the UHPLC-IMS-HRMS instrumental methodology can be found in [17].

2.2.1.2. UHPLC-MS/MS QqQ

The sample extracts were also analyzed for the qualitative determination of hormones, PFASs and other organic micropollutants by a DIONEX UltiMate 3000 UHPLC system (Thermo Scientific, Waltham, MA, USA) coupled to a triple

quadrupole mass spectrometer (TSQ QUANTIVA, Thermo Scientific, Waltham, MA, USA).

A Kinetex® Biphenyl column (100 mm × 2.1 mm i.d., 2.6 µm particle size, Phenomenex) was used for chromatographic separation of organic micropollutants and PFASs compounds. The mobile phase consisted of Milli-Q water and methanol, both with 0.1% formic acid. Flow rate was 600 µL min^{-1} and run time was 16 min, with switched positive and negative electrospray ionization modes. Furthermore, an Acquity BEH C18 column (50 mm x 2.1 mm, 1.7 µm, Waters Corporation, Manchester, UK) was used for chromatographic separation of hormones. The mobile phase for hormone analysis consisted of Milli-Q water with 5 mM ammonium acetate and acetonitrile (ACN) at a flow rate of 500 µL min^{-1}. Run time was 15 min using switching positive and negative electrospray ionization modes.

Heated electrospray ionization (H-ESI) was used to ionize the target compounds. The spray voltage was set to static: positive ion 3.5 kV and negative ion 2.5 kV. Nitrogen (purity >99.999%) was used as sheath gas (50 arbitrary units), auxiliary gas (15 arbitrary units), and sweep gas (2 arbitrary units) and collision gas. The vaporizer was heated to 400°C and the capillary to 325°C.

A detailed information of the low resolution methodologies followed as well as target compounds evaluated can be found elsewhere [38].

2.2.2. Criteria for identification

For UHPLC-IMS-HRMS screening, the identification criteria and confidence levels proposed previously by Celma *et al.* were followed [17]. In brief, level 1 confirmation (target screening) requires mass accuracy of both precursor and fragment ions < 3 ppm, RT deviation < 0.1 min and CCS deviation < 2% in relation to the reference standard. For levels 2 and 3 (suspect screening) mass accuracy of precursor and fragments ions should be < 3 ppm from the potential molecular formula. For suspect candidates, RT and CCS were predicted using a model developed elsewhere [39,40] with an accuracy of < ± 2min for RT, < ± 4% CCS

deviation for protonated molecules and < ± 5.8% CCS deviation for deprotonated molecules.

For qualitative targeted screening by LC-MS/MS QqQ, RT agreement with the reference standard as well as the observation of the different *m/z* transitions for each analyte (with relative deviation of ratios between monitored transitions below ± 30%) and signal-to-noise (S/N) ratio ≥ 3 were required for the identification of the compound, as suggested elsewhere [41]. For comparison purposes, reference standards were injected alongside the samples in the same chromatographic sequence.

2.3. Quality assurance for chemical analysis

Method performance was assessed by spiking a set of 15 ILIS with different psycho-chemical properties in both, samples and procedural blanks, to account for extraction efficiency . Spiked ILIS were chosen based on a set of compounds developed to cover the whole chromatographic run (**Table 2**) [42].

ILIS were spiked at 40 ng L^{-1} in raw sample yielding an expected final concentration in extract injected into the LC-MS system of 100 µg L^{-1}. The extraction performance was qualitatively evaluated by screening the spiked ILIS by means of UHPLC-IMS-HRMS.

2.4. Biological analysis of samples

The whole set of 11 samples and the 2 procedural blanks were biologically analyzed to evaluate water quality with a panel of 8 different toxicity endpoints. Aryl hydrocarbon receptor (AhR), (ant)agonistic androgenicity (AR), (ant)agonistic estrogenicity (ER), oxidative stress response (Nrf2) and (ant)agonistic vitamin D receptor (VDR) reporter gene assays were implemented as they are appropriate indicators of quality of environmental water bodies. Results on bioanalysis are presented elsewhere [30]. Extraction for biological analysis was analogue to the one herein performed excepting for the fact that no ILIS was spiked and that the reconstitution solvent was changed to ethanol instead of H_2O to enable its application into alive organisms. Biological

analysis performance was evaluated by analyzing specific positive controls for each bioassay alongside the analysis of wetland samples.

A detailed description of the different bioassays applied and the methodology followed is available at Celma *et al*. [30].

3. Results and discussion

3.1. Method performance evaluation

Spiked compounds were investigated by means of UHPLC-IMS-HRMS to qualitatively evaluate the extraction performance. **Table 2** indicates whether compounds could be identified in the samples or they were not detected due to inefficient recovery from sample treatment. Briefly, 60% of the spiked compounds were recovered and detected by means of IMS-HRMS in negative ionization mode and 83% in positive ionization mode. Interestingly, two of the

Table 2. Compounds spiked for quality control purposes. Detection of compound is indicated as '✓', no detection indicated as '-'.

	QC Compound	Proced. blanks		Wetland samples										
		A	B	1	2	3	4	5	6	7	8	9	10	11
ESI-POS	Ecgonine-d_3	-	-	-	-	-	-	-	-	-	-	-	-	-
	Morphine-d_3	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Methylone-d_3	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Norfloxacine-d_5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	MDPV-d_8 [a]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Venlafaxine-d_6	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Salicylic acid-d_4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	25-B-NBOMe-d_3 [b]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Ethofumesate-d_5	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Tebuconazole-d_6	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	THC-COOH-d_3 [c]	✓	-	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	DEHP-d_3 [d]	-	-	-	-	-	-	-	✓	-	-	-	-	-
ESI-NEG	Ecgonine-d_3	-	-	-	-	-	-	-	-	-	-	-	-	-
	Ethylparaben-d_4	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Irbesartan-d_6	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Ibuprofen-d_3	-	-	-	-	-	-	-	-	-	-	-	-	-
	THC-COOH-d_3 [c]	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

[a] MDPV-d_8: methylendioxypyrovalerone-d_8
[b] 25-B-NBOMe-d_3: 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine-d_3
[c] THC-COOH-d_3: 11-Nor-9-carboxy-Δ^9-tetrahydrocannabinol-d_3
[d] DEHP-d_4: di(2-ethylhexyl)phthalate-d_4

compounds that were systematically not detected in spiked samples were ecgonine-d_3 and di(2-ethylhexyl)phthalate-d_4. Those are the first and last compound eluting from the chromatographic column, denoting high and low polarity, respectively. Despite that they were lost during sample treatment, it is not expected to notably affect the overall performance of the screening as the wide majority of compounds included in this study are not of extreme polarities. Also, ibuprofen-d_3 was not observed in any of the spiked samples which might indicate potential degradation due to its reduced stability in environmental aquatic samples [43].

3.2. Wide-scope screening of organic micropollutants

3.2.1. Target screening

Target screening permitted the identification of 88 substances in the whole set of surface water bodies. **Table 3** summarizes the main findings, including the level of confidence of the identification. Those peaks appearing in procedural blanks at similar intensities than in wetland samples were excluded from further analysis.

From the 57 compounds included in both, IMS-HRMS and QqQ methods, 28 could be identified in the samples by at least one of the methodologies, yielding a total of 160 positives. While 43 were confirmed by both methods, 113 were identified only by MS/MS QqQ and 4 only by IMS-HRMS. **Table 3** shows that several positives could only be found by QqQ, due to its higher sensitivity, illustrating the advantage of using QqQ for detection and identification of target compounds when they are present at very low concentrations. In some cases, slight deviations in RT or mass accuracy were observed in IMS-HRMS screening, and therefore confirmation via MS/MS QqQ gathered was much useful. Besides, 23 more compounds (131 positives) monitored only by QqQ, and 37 compounds (127 positives) monitored only by IMS-HRMS, could also be identified. Independently from the instrumentation used, confirmations with IMS-HRMS or QqQ are considered with the same level of confidence for discussion purposes.

Table 3. Target compounds identified in water bodies from Spanish Mediterranean coastline. '✓' denotes confirmation at Level 1 by either UHPLC-IMS-HRMS or UHPLC-MS/MS QqQ. '✓* (RT)', '✓* (MS frag.)' and '✓* (MS)' denote confirmation at Level 1* via UHPLC-IMS-HRMS followed by an indication of the deviation observed.

Compound	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**	**#8**	**#9**	**#10**	**#11**
	Ebro River	**'Clot' Ebro**	**Peñíscola Lagoon**	**Peñíscola Channel**	**'Prat de Cabanes'**	**'Marjal' Pego-Oliva**	**Albufera Portet Sollana**	**Albufera Tancat Pipa'**	**Albufera 'Golf de Pujol'**	**Almenara**	**'Clot' Borriana**
Compounds only monitored by UHPLC-IMS-HRMS											
2-hydroxy-atrazine	✓* (RT)[a] (MS frag.)[b]	✓* (RT)					✓* (RT) (MS frag.)		✓* (RT) (MS frag.)	✓	
2-hydroxy-terbuthylazine	✓* (RT)								✓* (RT)	✓	✓
4-Acetamidoantipyrin	✓	✓					✓	✓	✓	✓	✓
4-formylaminoantipyrine	✓	✓					✓	✓	✓		
Atrazine	✓									✓	✓* (MS frag.)
Azoxystrobin	✓	✓				✓* (MS)[c]	✓	✓	✓	✓* (MS frag.)	
Bentazone	✓	✓					✓	✓	✓	✓	
Benzophenone-3			✓	✓							
Benzoylecgonine	✓	✓	✓* (MS)					✓			
Bromacil											✓* (MS)
Carbendazim	✓	✓* (RT)					✓* (RT)				✓* (MS)
Chlorfenvinphos			✓* (MS)	✓* (MS frag.)							
Clopidogrel carboxylic acid	✓* (MS)						✓* (MS)				
Deethyl-atrazine	✓* (MS frag.)						✓* (MS)		✓	✓	✓
Deethyl-terbumeton							✓* (RT)		✓* (RT)	✓	✓* (RT)

Compound	#1 Ebro River	#2 'Clot' Ebro	#3 Peñíscola Lagoon	#4 Peñíscola Channel	#5 'Prat de Cabanes'	#6 'Marjal' Pego-Oliva	#7 Albufera Portet Sollana	#8 Albufera Tancat Pipa'	#9 Albufera 'Golf de Pujol'	#10 Almenara	#11 'Clot' Borriana
Deisopropyl-atrazine										✓* (MS frag.)	✓* (MS frag.)
Desethyl terbuthylazine							✓		✓	✓	✓
Diflufenican											✓
Flumequine		✓									
Imazalil	✓* (RT)		✓	✓			✓* (RT)				✓
Iomeprol	✓							✓			
Isoproturon	✓* (MS frag.)										
Lincomycin	✓							✓* (RT)	✓		
Lorazepam	✓							✓* (MS)			
Losartan Carboxylic acid	✓* (MS frag.)						✓	✓* (MS)	✓* (MS frag.)		
MCPA							✓* (MS frag.)	✓* (MS)	✓* (MS frag.)		✓
Metalaxyl	✓						✓		✓	✓	
Metolachlor	✓										
Propamocarb	✓		✓	✓		✓			✓	✓	
Propiconazole	✓	✓	✓				✓* (MS frag.)	✓* (MS frag.)	✓* (MS frag.)		✓* (MS)
Propyzamide	✓										
Prosulfocarb	✓										
Simazine							✓		✓* (MS frag.)	✓	✓* (MS frag.)
Sulfamethazine	✓										
Tebuconazole	✓	✓			✓	✓* (MS)	✓	✓	✓	✓	✓

Compound	#1 Ebro River	#2 'Clot' Ebro	#3 Peñíscola Lagoon	#4 Peñíscola Channel	#5 'Prat de Cabanes'	#6 'Marjal' Pego-Oliva	#7 Albufera Portet Sollana	#8 Albufera Tancat Pipa'	#9 Albufera 'Golf de Pujol'	#10 Almenara	#11 'Cloť Borriana
Terbumeton										✓	
Terbuthylazine	✓									✓	✓
Compounds only monitored by UHPLC-MS/MS QqQ											
10,11-Dihydro-10-hydroxycarbamazepine	✓										
17α-Estradiol		✓			✓						
Acesulfame	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Amidotrizoic acid	✓	✓					✓	✓	✓	✓	✓
Bisoprolol	✓						✓	✓	✓		
Caffeine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Cetirizine	✓						✓	✓	✓		
Citalopram			✓	✓			✓	✓	✓		
DEET (N,N-diethyl-m-toluamide)	✓	✓			✓	✓	✓	✓	✓		
Diazepam							✓	✓	✓		
Diltiazem	✓							✓			
Fluconazole		✓					✓	✓	✓		
Hydrochlorothiazide	✓						✓	✓	✓	✓	✓
Loratadine	✓										
Memantine							✓	✓	✓		
Mirtazapine	✓										
Nicotinamide	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Nicotine	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Niflumic acid							✓	✓	✓		
Oxazepam	✓						✓	✓	✓		
Panthenol			✓	✓							

Compound	#1		#2		#3		#4		#5		#6		#7		#8		#9		#10		#11	
	Ebro River		'Clot' Ebro		Peñíscola Lagoon		Peñíscola Channel		'Prat de Cabanes'		'Marjal' Pego-Oliva		Albufera Portet Sollana		Albufera Tancat Pipa'		Albufera 'Golf de Pujol'		Almenara		'Clot' Borriana	
Theophylline	✓		✓		✓		✓		✓		✓		✓		✓		✓		✓		✓	
Valsartan	✓		✓		✓		✓						✓		✓		✓		✓		✓	
Compounds included in both UHPLC-IMS-HRMS and UHPLC-MS/MS QqQ analyses																						
	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ	HR MS	QqQ
10,11-epoxy-carbamazepine		✓												✓		✓		✓		✓		
Amoxicillin		✓		✓										✓		✓		✓		✓		
Azithromycin		✓				✓		✓		✓				✓		✓		✓		✓		✓
Benzophenone-4	✓	✓											✓	✓	✓	✓	✓	✓				
Carbamazepine	✓	✓		✓		✓		✓						✓	✓	✓	✓* (MS)	✓		✓		✓
Clarithromycin		✓												✓		✓		✓		✓		✓
Clindamycin		✓		✓										✓		✓		✓				
Clopidogrel		✓																				
Cocaine			✓* (MS)				✓* (RT)															
Codeine		✓												✓		✓		✓				
Estrone		✓		✓						✓		✓		✓		✓		✓				✓
Iopromide	✓																					
Irbesartan		✓		✓									✓* (MS frag)	✓	✓* (MS)	✓		✓		✓		✓
Lidocaine		✓		✓														✓				✓
Losartan	✓	✓											✓* (MS)	✓	✓* (MS)	✓		✓				
Metronidazole		✓																				

Compound	#1		#2		#3		#4		#5		#6		#7		#8		#9		#10		#11	
	Ebro River		'Clot' Ebro		Peñíscola Lagoon		Peñíscola Channel		'Prat de Cabanes'		'Marjal' Pego-Oliva		Albufera Portet Sollana		Albufera Tancat Pipa'		Albufera 'Golf de Pujol'		Almenara		'Clot' Borriana	
O-Desmethyl venlafaxine	✓	✓		✓									✓* (MS frag)	✓		✓		✓	✓* (MS frag)	✓		
Ofloxacin		✓				✓		✓						✓		✓		✓				✓
Omeprazole		✓																				
Phenazone															✓							
Primidone		✓		✓										✓		✓		✓		✓		✓
Salicylic acid		✓		✓		✓		✓		✓		✓		✓		✓		✓		✓		✓
Sulfamethoxazole	✓	✓		✓		✓		✓						✓		✓		✓		✓		✓
Telmisartan		✓		✓						✓				✓		✓		✓		✓		
Thiabendazole	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓	✓* (RT)	✓
Tramadol		✓												✓		✓		✓		✓		✓
Trimethoprim		✓																				
Venlafaxine	✓	✓											✓	✓		✓	✓	✓	✓	✓		✓

[a] ✓* (RT): Confirmation at Level 1 with a slight deviation in RT from standard.

[b] ✓* (MS frag.): Confirmation at Level 1 with a slight deviation in mass accuracy of fragment ions.

[c] ✓* (MS frag.): Confirmation at Level 1 with a slight deviation in mass accuracy in (de)protonated molecule.

The most prevalent compounds were pharmaceuticals and their metabolites with 43 and 10 substances identified, respectively. 25 pesticides and 6 transformation products, 2 hormones and 2 personal care products were also identified. Considering that the set of surface water samples studied had no direct impact of wastewater streams except for 'Ebro' River estuary and Albufera Natural park, it is remarkable that the most dominant group of chemicals were pharmaceuticals. Yet, the figures of pesticides identified were expected due to agricultural practices in the surrounding areas.

Several compounds (*acesulfame, caffeine, nicotine, salicylic acid, theophylline,* and *thiabendazole*) were identified in all samples. Most of them are markers of human activity: *acesulfame* (sweetener), *caffeine* (main stimulant from coffee and tea), *nicotine* (main stimulant from tobacco), *salicylic acid* (important active metabolite of aspirin), and *theophylline* (metabolite from caffeine) [44]. *Thiabendazole*, however, is a pesticide mainly used in fruit and vegetable agriculture due to its antifungal properties although it can also be used as a pharmaceutical against fungal infections [45,46]. Contrarily, several compounds were only identified once: 10 pharmaceuticals (*clopidogrel*, *flumequine, iopromide, loratadine, metronidazole, mirtazapine, omeprazole, phenazone, sulfamethazine* and *trimethoprim*) and 7 pesticides (*bromacil, diflufenican, isoproturon, metolachlor, propyzamide, prosulfocarb,* and *terbumeton*). The number of identified chemicals by combination of IMS-QTOF MS and MS/MS QqQ methodologies highlight the importance of a carefully thought out strategy for wide scope screening campaigns. It is also remarkable that while prior studies reported PFASs in surface water bodies using similar extraction procedures [47,48], none of them (n = 10) was found in any wetland sample herein studied (instrumental limits of detection in sub-ng L^{-1} level [49]).

From the sample perspective, the sample where most contaminants were identified by target analysis was #1 'Ebro' river estuary (68 compounds) followed by #9 Albufera – 'Golf de Pujol' (54 compounds), #7 Albufera – 'Portet Sollana' (53 compounds) and #8 Albufera – 'Tancat de la Pipa' (49 compounds). This could be expected due to the fact that these locations are affected by urban wastewater effluents. The 'Ebro' river collects effluent wastewater from

different big cities (*e.g.* Zaragoza ~666,000 inhabitants- and Amposta ~20,000 inhabitants-) and, Albufera Natural Park is affected by wastewater effluents and street run-off streams from Valencia (~790,000 inhabitants). Therefore, finding pharmaceuticals and metabolites in these locations is not surprising. Similarly, the presence of different pesticides due to nearby agricultural activities was predictable. Previous studies conducted in the Ebro river and/or Albufera Natural Park also found similar contamination pattern by pharmaceuticals and pesticides [6–8]. Contrarily, samples #5 'Prat de Cabanes' and #6 'Marjal' Pego-Oliva showed the lowest number of identified target chemicals, 13 and 12, respectively. The latter was also in agreement with previous studies [50].

3.2.2. Suspect screening

A suspect screening of more than 900 substances was performed by means of UHPLC-IMS-QTOF MS. During a first data revision process, mass spectrometric features were required to show intensity above 2,000 counts for positive and negative ionization modes. Also, chromatographic peak width < 0.4 min, mass error from candidate structure below 3 ppm ((de)protonated molecule), and the presence of at least one fragment ion was required [17]. Under these conditions, 77 candidate compounds were indicatively identified to be present in the samples. In a more exhaustive data revision, high collision energy spectra were inspected individually and compared to data reported in scientific literature and mass spectral databases such as Mass Bank of North America and Mass Bank Europe [51,52]. In those cases where no previously reported data was available, mass fragmentation was evaluated using MassFragment tool within UNIFI [53] for the finding of diagnostic fragments supporting the identification. In order to improve the identification process, chromatographic RT and CCS values were predicted for the suspected structures and compared with the empirical measurements [39,40]. Accuracy for predicted values was considered as satisfactory for RT (below ± 2 min) and for CCS (below ± 4% for protonated molecules and ± 5.8% for deprotonated molecules).

At this point, the number of suspected substances was reduced to 27 candidate compounds. Among them, *17-α-estradiol*, *benzophenone*, *caffeine*, *citalopram*,

nicotine, *niflumic acid*, *oxazepam* and *valsartan* (**Table 3**) could be afterwards confirmed by UHPLC-MS/MS QqQ analysis. The remaining 19 substances, tentatively identified at Level 2a or Level 3, are shown in **Table 4** (7 pesticides and 2 TPs, 3 pharmaceuticals and 6 metabolites and 1 hormone metabolite). Reference standards could be purchased for 15 substances except for *ethynyl estradiol 3*-β-*D-glucuronide*, *3-[(4-Carboxy-4-methylpentyl)oxy]-4-methylbenzoic acid*, *nordiazepam* and *simvastatin acyl*-β-*D-glucuronide*. 3 compounds (*3,4-dichloroaniline, amisulpride and cotinine*) could be, then, confirmed by reference standards and 12 tentative identifications were discarded as also indicated in **Table 4**. Although the identity of the latter could not be explained in terms of the proposed structures, unidentified substances were detected over the different samples. Further studies could be conducted towards the identification of those particular features.

Table 4. Suspect compounds identified at Level 2a or Level 3 based on different evidences gathered (diagnostic fragments -Diag. frag-, literature review, or data available at Mass Bank of North America -MoNA- or Mass Bank Europe -MBEU-).

Compound	#1 Ebro River	#2 'Clot' Ebro	#3 Peñíscola Lagoon	#4 Peñíscola Channel	#5 Prat de Cabanes	#6 'Marjal' Pego-Oliva	#7 Albufera Portet Sollana	#8 Albufera 'Tancat Pipa'	#9 Albufera 'Golf de Pujol'	#10 Almenara	#11 'Clot' Borriana	Evidence	Identity confirmed by reference standard?
3,4-dichloroaniline								Lev. 2a				Literature	Yes, at Lev. 1
8-OH quinoline		Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a	MoNA; MBEU	No
Amisulpride	Lev. 2a						Lev. 2a	Lev. 2a	Lev. 2a	Lev. 2a		Literature	Yes, at Lev. 1
Chlorotoluron	Lev. 2a											MBEU	No
Cotinine	Lev. 2a	Lev. 2a					Lev. 2a	Lev. 2a	Lev. 2a		Lev. 2a	Literature	Yes, at Lev. 1
Eth-Estr. 3-β-D-Gl.[a]											Lev. 3	Diag. frag.	*n.a.*
Gemfibrozil Metab.[b]	Lev. 3							Lev. 3			Lev. 3	Diag. frag.	*n.a.*
Metolachlor ESA[c]	Lev. 2a	Lev. 2a										Literature	No
Metoxuron	Lev. 2a											Diag. frag.; Lit.	No
Monuron	Lev. 2a											Diag. frag.; Lit.	No
Nordiazepam							Lev. 2a	Lev. 2a				Literature	*n.a.*
Pirbuterol											Lev. 3	Diag. frag.	No
Pravastatin Lactone		Lev. 3			Lev. 3							Diag. frag.	No
Prometon							Lev. 2a			Lev. 2a		MoNA; MBEU	No
Pyrimethanil							Lev. 2a		Lev. 2a			Literature	*n.a.*
Simvas. Acyl-β-D-Gl.[d]							Lev. 3		Lev. 3			Diag. frag.	*n.a.*
Sitagliptin								Lev. 2a				Literature	No
Trietazine										Lev. 3		Diag. frag.	No
Vildagliptin	Lev. 2a						Lev. 2a	Lev. 2a	Lev. 2a			Literature	No

[a] Eth-Estr. 3-β-D-Gl.: Ethynyl Estradiol 3-β-D-Glucuronide; [b] Gemfibrozil Metab.: 3-[(4-Carboxy-4-methylpentyl)oxy]-4-methylbenzoic acid; [c] Metolachlor ESA: Metolachlor ethanesulfonic acid;[d] Simvas. Acyl-β-D-Gl.: Simvastatin Acyl-β-D-glucuronide

3.3. Toxicological fingerprint as a complementary tool of chemical analysis for a comprehensive water quality evaluation

Evaluation of water quality only by means of chemical analysis has limitations as only a fraction of all potentially toxic compounds is under the radar. Therefore, recent strategies have been proposed to combine both chemical and bioanalytical analyses to complement the results obtained [24,27,29]. In this work, information gathered from chemical and biological analyses have been combined with the aim of linking observed toxicities with chemicals identified in samples [30].

Briefly, toxicological evaluation of wetland samples consisted of 8 toxicity endpoints: aryl hydrocarbon receptor (AhR), androgen receptor (AR) activation and inhibition, estrogen receptor (ER) activation and inhibition, oxidative stress response and vitamin D receptor (VDR) activation and inhibition. The vast majority of samples, except for #10 'L'Estany Almenara' and #11 'Clot Borriana', showed a clear activation of the AhR endpoint, mainly triggered by the presence of aromatic hydrocarbon chemicals. Therefore, the AhR activity observed for the majority of samples is most likely explained by the elevated number of organic compounds identified with aromatic hydrocarbon groups within their chemical structure. However, contributions for the overall AhR activity from undetected compounds could also occur. For the remaining toxicity endpoints evaluated, apart from sporadic detections, quantifiable estrogenic inhibition was observed for samples #8 Albufera – 'Tancat la Pipa' and #9 Albufera – 'Golf de Pujol' which are potentially affected by wastewater and street run-off water streams from Valencia (Spain).

The only compound exclusively detected in samples #8 and #9 was *phenazone*. Potential for inhibition of estrogenic receptor was modelled through the ToxCast Pathway Model within CompTox chemicals dashboard (https://comptox.epa.gov/dashboard) as described by Browne *et al*. [54]. However, *phenazone* was found to be inactive for the antagonistic ER and, therefore, the inhibition of such toxicity endpoint cannot be explained in terms of this unique compound. Consequently, individual antagonistic estrogenicity for

the whole set of chemicals identified in the samples were predicted (either active or inactive) by means of the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) [55] integrated within OChem website (Online Chemical Database with modelling environment, www.ochem.eu, model 285) [56]. In total, 16 substances were found to be estrogenic inhibitors. Several of these compounds were found in samples #8 and/or #9 (*azithromycin, benzophenone-4, bisoprolol, cetirizine, citalopram, clarithromycin, estrone, gemfibrozil M3 (3-[(4-Carboxy-4-methylpentyl)oxy]-4-methylbenzoic acid), o-desmethylvenlafaxine, simvastatin acyl-β-D-glucuronide, tramadol* and *venlafaxine*). Therefore, the observed activity in those samples could be partially explained by the presence of these compounds and also due to a potential synergic mixed effect to enhance the antagonistic estrogenicity of the sample. Nonetheless, thousands of sample components still remain unidentified and might be playing an important role for the toxicity levels observed in samples #8 and #9. Besides, some of these estrogen receptor antagonistic compounds were also detected in non-active samples. Yet, the detected signals were in general lower than in active samples and, therefore, not concentrated enough to trigger estrogenic antagonism. Similarly, there were also other predicted antagonistic compounds that were not identified in samples #8 and #9 but in other samples (*17α-estradiol, benzophenone-3, diflufenican and ethynyl estradiol 3-β-D-glucuronide*). Although these compounds were found in non-active samples, their concentration might be not high enough to be detected by the bioanalytical tools applied.

An exhaustive, meaningful and complete chemical explanation of the toxicity figures observed would have required the knowledge of the identity of all compounds present in the sample that can lead to those particular effects. However, this is nearly unachievable due to different implicit limitations of chemical analysis. The chemical space covered in screening strategies is mainly limited by the sample extraction procedure as well as the separation technique selected for analysis. In this study, extracts for chemical and biological analysis were obtained identically to minimize the impact of sample treatment when it comes to compare chemical and biological analyses. However, the space covered during chemical analysis was limited to LC-amenable compounds analyzed

under reverse phase LC and, therefore, some toxicity drivers in the analyzed wetland samples can still remain undiscovered. It is expected that the utilization of additional separation techniques (*e.g.* gas chromatography or hydrophilic interaction chromatography) as well as the inclusion of larger target and suspect screening databases would yield a higher rate of identified compounds. Therefore, more insight about the actual composition of wetland samples can be gathered and, thus, might be helpful to better explain the observed toxicities. Nonetheless, it is not realistic in terms of resources and time-efforts to seek for the identification of the whole universe of chemicals that can be present in an environmental sample.

The results herein presented highlight the necessity of applying combined strategies to give a more comprehensive insight in the quality of water. In summary, different chemicals were found potentially responsible for the estrogenic toxicities observed although still unidentified compounds can be playing an important role. Although the solely presence of the identified chemicals might not arise any concern during chemical analysis, the integration of biological results into the water quality monitoring strategy remarks how combined effects of organic micropollutants might threaten the ecosystem.

4. Conclusions

A comprehensive target and suspect screening of > 1,500 organic micropollutants has been conducted by the combined application of UHPLC-IMS-QTOF MS and UHPLC-QqQ screening. Different benefits from each technique have been gathered *i.e.* cleaner mass spectral and the additional identification parameter provided by IMS-HRMS as well as the increased sensitivity obtained by means of UHPLC-LRMS. As a result, 91 different organic micropollutants were confirmed at the most confident level of identification (Level 1), and 5 were tentatively identified at Levels 2a or 3 in the set of wetland samples studied. Several positives could only be found by UHPLC-QqQ, due to its higher sensitivity, illustrating the advantage of using QqQ for detection and identification of target compounds when they are present at very low concentrations. Pharmaceuticals and pesticides were the most prevalent groups of chemicals detected. Additionally, a complete picture of the quality of the water bodies has been evaluated by putting into context chemical analysis and bioanalytical quality assessment. 12 organic micropollutants with predicted antagonistic estrogenic activities have been found in active samples. Yet, other still unidentified chemicals can be contributing to the overall activity of the sample. Also, the elevated number of compounds identified with aromatic hydrocarbon groups could likely be an explanation for the AhR activities observed. However, the limited chemical space covered by chemical analysis still stands in the way for a complete and meaningful chemical explanation of measured biological activities. Although identifying an organic micropollutant in a water sample might not be of concern, the integration of biological results into the water quality monitoring strategy highlighted how mixed effects might really threaten the ecosystem quality. That remarks the importance of combining chemical analysis with bioanalytical tools to provide an improved overview of the actual status of water quality in natural reservoirs**.**

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4.3. Discussion

Water quality evaluation by means of chemical analyses have been widely investigated and several efforts have been devoted to the development, application and standardization of analytical strategies to monitor OMPs (Gago-Ferrero et al., 2020; Hernández et al., 2015; Krauss et al., 2010; Menger et al., 2020). However, they may give a biased insight about the real status of the water sample due to the implicit limitations of the methodology applied (compound amenability for chromatographic separation, MS detection, data processing, etc.). Similarly, biological analyses are able to provide an overall observation of the toxicity of the sample for certain endpoints and, therefore, reveal biological activities that could be relevant to adverse effects in freshwater organisms (Altenburger et al., 2019; Brack et al., 2016; Dopp et al., 2019). Yet, bioassays cannot identify the compounds responsible for the observed activity. Thus, no comprehensive water quality monitoring can be implemented based on biological analysis only (Dopp et al., 2019; Escher et al., 2014).

Recently proposed effect-directed analysis (EDA) has revolutionized the monitoring of OMPs by both identifying chemicals and their associated risk for the surrounding organisms (Hernández et al., 2019). The integration of both biological and chemical analyses can, therefore, measure mixed toxic effects of

compounds as well as to potentially relate them with detected organic micropollutants (Brunner et al., 2020; Di Paolo et al., 2016; Lundqvist et al., 2019b; Tousova et al., 2017). However, there is still many aspects to be wisely considered in EDA approaches:

i. ***Problem formulation, study design and selection of bioassays.*** EDA is a highly resource- and time-consuming approach. Therefore, problem formulation and study design should be carefully decided in order to optimize the efforts invested (Altenburger et al., 2019). Highly important is the choice of appropriate endpoints to evaluate. The selection should be based on prior effects seen in the field as well as on possible pollution sources, on-site specific contaminants or toxicants that might cause an adverse effect for the surrounding ecosystem (Brack et al., 2016). In this sense, the panel of 5 different bioassays implemented in **scientific article 6** (AhR, AR, ER, VDR and Nrf2) were selected based on their potential to detect combined effects of OMPs such as alterations of the normal functioning of freshwater organisms due to elevated presence of aromatic aryl hydrocarbons or endocrine disrupting chemicals.

ii. ***Sampling strategy and sample treatment.*** EDA aims to address the whole complex mixture of sample components rather than individual chemicals. Thus, sample collection and extraction procedures should be developed in a way that minimizes loss of mixture components (Brack et al., 2016). Water samples can be collected either as a grab sample or composite sample, although the latter is often preferred due to its higher representativeness of temporal changes of the water body. Grab samples, as collected in **scientific article 6** and **7,** are generally picked up due to their easiness of collection. Especially since large volume of samples are often necessary to perform bioanalysis (Altenburger et al., 2019). After collection and in order to enable the bioanalysis of samples, water samples need to be preconcentrated, which is often performed by means of SPE procedure. However, this step may alter the original composition of the sample depending on the characteristics of the SPE

sorbent selected (Altenburger et al., 2019; Hernández et al., 2019; Menger et al., 2020). Yet, sample extract may only represent part of the original composition and some of the compounds present in the original water sample may not be found in the extract. Although this is not only a limitation of biological analysis but also for chemical analysis, the latter does not necessarily require preconcentration due to its higher sensitivity. Commonly, generic extraction procedures are recommended for EDA strategies. Consequently, studies presented in **scientific article 6** and **7** made use of generic SPE extraction procedure by means of HLB sorbent. Recently, mixed sorbents with different chemical characteristics have been elsewhere tested in order to cover a wider range of compounds and better represent the original composition of the sample (Gago-Ferrero et al., 2020). In addition, passive sampling might also represent a good alternative for sample collection and extraction as it combines both large sampling volumes and integrative extraction of sample components for a long period of time (Brack et al., 2016).

iii. ***Fractionation.*** In the cyclic workflow of EDA, sample fractionation represent a key step for an appropriate performance of the strategy. By separating the whole sample extract into smaller aliquots, sample complexity is reduced and the strategy can be more effectively directed towards the identification of the toxicity drivers. With fractionation often based on preparative chromatographic separation (and mainly LC), special attention should be drawn to the toxicity recovery and absence of blank toxicity, the selection of appropriate solvents as well as the orthogonal separation techniques for successive fractionation steps (Brack et al., 2016). However, no fractionation could be applied for the studies presented in **scientific article 6** and **7** due to the fact that no fractionator was available at the laboratory. Yet, it is expected that further analysis including fractionation would have allowed a higher confidence in the identification of toxicity drivers performed in **scientific article 7**.

iv. ***Standardized protocols.*** Similarly to chemical analysis, bioanalysis results strongly depends on the methodology applied for the assay. A simple search in the scientific literature reveals several different approaches to evaluate activity over similar endpoints, but with different experimental setups. As a consequence, results obtained with different methodologies are hardly comparable. In this regard, standardized protocols help to achieve results that permit the intercomparison between different studies. As an example, the evaluation of AR and ER response have been regulated by means of different protocols by the Organization for Economic Cooperation and Development (OECD) (OECD, 2016, 2012). Thus, results obtained following the recommended guidelines can be compared with third-party data. Hence, it is expected that future standardization of protocols for the evaluation of water quality by means of bioanalysis of relevant toxicity endpoints will further expand the applicability of EDA approaches. Biological analysis of wetland samples in **scientific article 6** was conducted following standardized protocols for AR and ER. However, AhR, Nrf2 and VDR bioassays were performed based on previously in-house developed methodologies.

v. ***Effect-based trigger values***. Effect-based trigger values (EBT) can be used as a means to evaluate the potential hazards and implications of the bioactivities observed in a panel of *in vitro* bioassays. Briefly, EBT can be understood as a threshold level indicating the value of minimum activity which causes concern for a particular endpoint (Alygizakis et al., 2019; Been et al., 2021; Escher et al., 2018). However, EBTs are still being developed and there is no scientific consensus about them since their value relies on many different particularities of the method used such as the bioassay used, the positive control to interpolate bioequivalent concentrations or the endpoint for which have been developed (Lundqvist et al., 2019a). Consequently, it was decided that no EBTs were going to be used in the **scientific article 6** and **7**.

vi. ***Limited information on toxicity of individual OMPs***. The final goal of implementing EDA strategies for the monitoring of water quality is the

identification of chemicals responsible for potential hazardous bioactivities observed in the sample (Altenburger et al., 2019; Brack et al., 2016). However, there is still a limited amount of information available about the toxicity of individual OMP over relevant endpoints. This strongly limits the real applicability of complete EDA approaches in environmental samples since it is often difficult to link observed OMP with empirical bioactivities. Thus, *in silico* prediction of toxicities based on molecular descriptors can be of extraordinary help to complement the empirical observations of both chemical and biological analyses (Mansouri et al., 2016; Williams et al., 2017). In the particular case of **scientific article 7**, individual estrogenic activity of observed OMP was consulted in CompTox Chemicals Dashboard (https://comptox.epa.gov/dashboard) from the Environmental Protection Agency (EPA, US) by means of the Pathway Model as described by Browne *et al.* (Browne et al., 2015). However, no data was available for the compounds of interest and *in silico* prediction through the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) (Mansouri et al., 2016) integrated within OChem website (Online Chemical Database with modelling environment, www.ochem.eu, model 285) (Sushko et al., 2011) was necessary. This permitted the connection between identified chemicals in **scientific article 7** and observed antagonistic ER toxicity in **scientific article 6**.

vii. ***Potential mixed effects***. Environmental samples are complex mixtures of several chemicals from different families. Therefore, the activity measured by bioanalysis responds to the overall sample mixture as a sum of the individual toxicities of each compound present in the samples (Brack et al., 2016). In this sense, it is possible that, due to their additive behavior, individual effects can compensate each other resulting in a total non-active mixture. Differently, a synergistic effect can be observed when the total effect is higher than the sum of individuals. Also, antagonistic effect occurs when the total effect of the chemical mixture is less severe than the sum of individuals. Although, at this point, it is very difficult to hypothesize about combined effects, they might occur

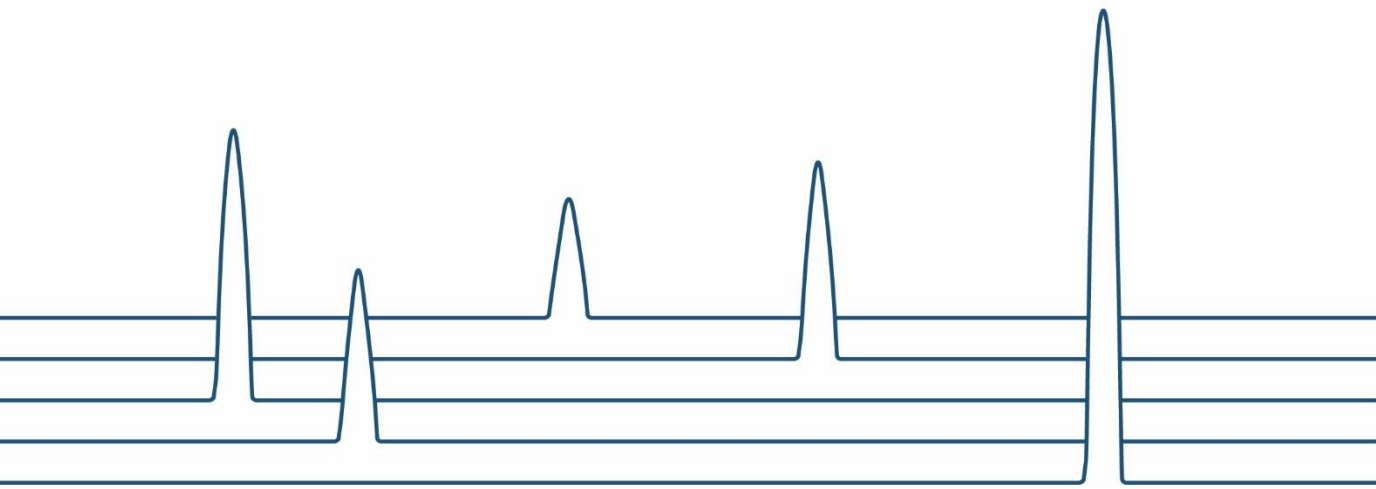
and, therefore, further studies need to be conducted to give some light on the actual extent of this phenomenon.

viii. ***Toxicity driver identification***. The identification of compounds responsible for the detected bioactivity is challenging and often requires the combination of TS, SS and NTS. At first, TS and SS of compounds based on individual toxicity data should be carried out as it was performed in **scientific article 7**. However, due to the abovementioned limited information of individual toxicities, NTS might also be necessary to identify the chemical or mixture of chemicals triggering a particular toxicity endpoint. Additionally, the combination of different types of chemical analysis such as LC or GC may increase the likelihood of toxicity driver identification.

As highlighted on the previous pages, there are several aspects that are key for a proper application of EDA strategies for monitoring water quality. The reason behind relies on the fact that EDA is a highly multidisciplinary approach which combines environmental analytical chemistry, ecotoxicology and computational sciences (Altenburger et al., 2019; Brack et al., 2016; Hernández et al., 2019). Therefore, it is expected that singular advances in those particular sciences will foster the implementation of EDA approaches for the monitoring of ecosystem quality. Additionally, the development of robust and efficient EDA approaches will increase the ecological relevance of their findings and allow environmental decision and policy makers have a more comprehensive insight about the actual scenario of relevant water bodies.

CHAPTER 5.

MONITORING NEW PSYCHOACTIVE SUBSTANCES CONSUMPTION BY URINE AND WASTEWATER ANALYSIS



Chapter 5. Monitoring New Psychoactive Substances Consumption by Urine and Wastewater Analysis

5.1. Introduction

5.2. Scientific Articles

5.2.1. Scientific article 8.

"Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations"

Current Opinion in Environmental Science & Health 9 (2019) 1-12

5.2.2. Scientific article 9.

"Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology"

Journal of Chromatography A 1602 (2019) 300-309

5.2.3. Scientific article 10.

"Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial"

Analytica Chimica Acta 1145 (2021) 132-147

5.3. Discussion of Results

5.1. Introduction

New Psychoactive Substances (NPS) are defined by the United Nations Office on Drugs and Crime (UNODC) as chemical units that produce similar effects to those induced by illicit drugs (ID) and that they are not strictly regulated by international conventions (UNODC, 2013). In other words, NPS are compounds designed to mimic effects of illicit drugs, such as cocaine, cannabis and amphetamines, but evade law enforcement often by introducing only minor modifications to the chemical structures of controlled substances (EMCDDA, 2007). In 2021, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) notified the detection of 46 new NPS for the first time in 2020 which were included in the list of 830 NPS detected since 2008 (**Figure 5.1**) (EMCDDA, 2021). The number of substances appearing in the market seems to have decreased since 2014, when a maximum of new NPS were reported. **Figure 5.1** also shows the different chemical classes encompassed under the category of NPS, such as cathinones, cannabinoids, phenethylamines, opioids and tryptamines (EMCDDA, 2020). This illustrates how versatile and ever-changing the NPS market is.

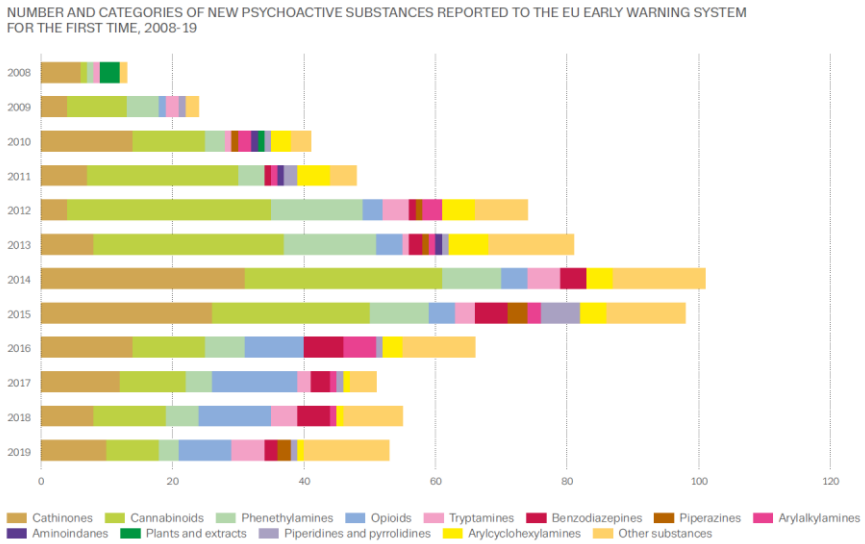


Figure 5.1. Number and categories of new psychoactive substances reported to the European Union Early Warning System for the first time (2008-2019). Extracted from European Drug Report 2020 (EMCDDA, 2020).

NPS are easily available to consumers mainly through e-commerce and have become a growing problem in many communities because they are, among others, responsible of several fatal intoxications (EMCDDA, 2016a; Krotulski et al., 2018; Walterscheid et al., 2014). The composition and purity of products containing NPS are often unknown, placing drug consumers at an even higher risk compared to well-known conventional ID (EMCDDA, 2019; UNODC, 2018). Hence, NPS are threatening public health and challenge professionals and policymakers in the identification, prevention, treatment and control policies (Hughes et al., 2018). Therefore, a comprehensive strategy to detect and monitor NPS at an early stage is pivotal. Apart from relevant sources of information such as surveys, seizures, or intoxications, WW analysis can provide reliable and almost real-time data about what is being consumed within a community (EMCDDA, 2016b).

Like any other substance consumed by humans, NPS are excreted through urine or faeces, either unchanged or as metabolites, ending up in the sewage network of a city. Wastewater-based epidemiology (WBE) relies on the analysis of

influent wastewater (WW) samples entering wastewater treatment plants (WWTP) to evaluate the population life-style habits or exposure to certain groups of chemicals (Gracia-Lor et al., 2017). In 2005, Zuccato *et al.* (Zuccato et al., 2005) explored, for the first time, WBE for the estimation of cocaine consumption in Italy. This approach was afterwards expanded to the analysis of several ID across different countries (Castiglioni et al., 2006; Zuccato et al., 2008) to the point that it has been established as an annual monitoring campaign of ID consumption in many European communities (González-Mariño et al., 2020; Ort et al., 2014). Sewage analysis CORe group Europe (SCORE, https://score-cost.eu), formed by several leading research groups across Europe, has devoted many efforts to harmonize the protocols and perform annual inter-laboratory exercises (Ort et al., 2010; van Nuijs et al., 2018). WBE (**Figure 5.2**) (Castiglioni et al., 2016, 2014) involves *(i)* the collection of representative 24-h composite WW samples at the inlet of the WWTP, *(ii)* calculation of daily mass loads of drug residues (μg day^{-1}) by multiplying measured concentrations of target residues (ng L^{-1}) with daily flow rates of influent WW (m^3 day^{-1}), *(iii)* estimation of total consumption through back-calculation by applying specific correction factors

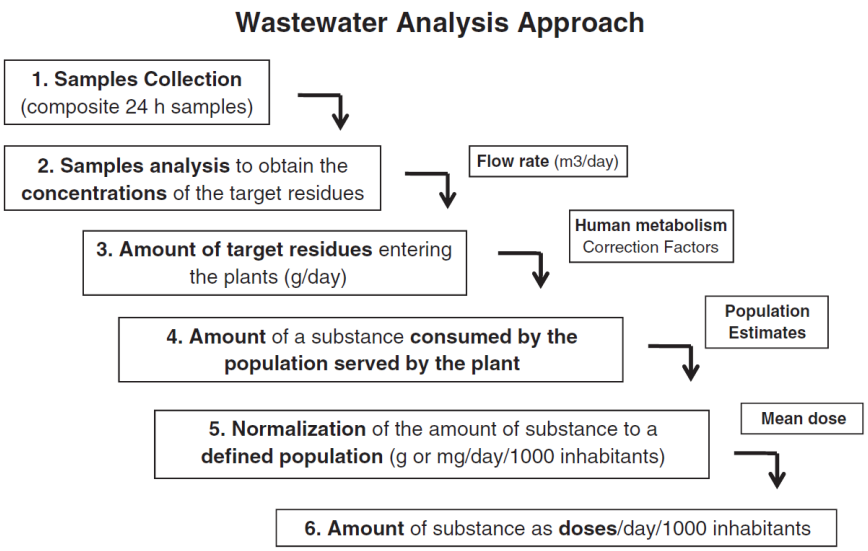


Figure 5.2. Main consecutive steps of the wastewater analysis approach and data required for estimation of substance use within a community. Extracted from Castiglioni et al. (Castiglioni et al., 2014).

that consider the average excretion rate for a given drug residue (van Nuijs et al., 2011; Zuccato et al., 2008), *(iv)* normalization of consumption data with population to facilitate comparison between cities (mg day^{-1} 1000 $inhabitants^{-1}$) and *(v)* the assumption of mean doses to recalculate consumption data in doses day^{-1} 1000 $inhabitants^{-1}$.

The application of WBE for the analysis of NPS has also been explored as the analysis of urban WW can provide an anonymized, comprehensive and objective picture on community-wide use (Archer et al., 2018, 2014, 2013; Bade et al., 2018, 2017; Bijlsma et al., 2020; Gracia-Lor et al., 2017; López-García et al., 2018). Alternatively, the analysis of pooled urine at special settings with higher likelihood of NPS consumption can also provide a realistic and anonymized picture of the NPS situation in a community (Baz-Lomba et al., 2016; Bijlsma et al., 2020; Hall et al., 2012; Hoegberg et al., 2018; Prichard et al., 2014). However, the application of WBE to NPS is not as straightforward as for ID. The low concentrations expected for this type of substances, the lack of metabolism studies and in-sewer stability studies makes it difficult to establish appropriate biomarkers and correction factors. Furthermore, the continuous appearance of new NPS together with the limited availability of commercial reference standards and the difficulties and bureaucracy to purchase them, make the development of quantitative methods difficult. Therefore, there is an increasing interest in developing and applying wide-scope qualitative screening strategies able to detect and identify a large number of NPS by means of HRMS using full spectrum acquisition modes (Bijlsma et al., 2020; Diamanti et al., 2019; Salgueiro-González et al., 2019). This analytical approach can be combined with large and collaborative databases and strategic sampling of both WW and PU at *e.g.* nightlife setting or special events. This will give a more comprehensive insight into the NPS consumption scenario. Additionally, new analytical developments providing extra sensitivity for targeted methodologies will allow to reach lower limits of detection for those substances for which reference standards are available. This permits to determine these NPS at low concentrations in WW samples. Finally, combining these two complementary techniques produce data that permit policymakers to take strategic decisions.

5.2. Scientific articles

The analytical strategies developed in previous chapters of this thesis were of help to overcome some of the major challenges of NPS monitoring in WW and PU samples. Hence by combining LRMS targeted analysis, HRMS suspect screening using large NPS databases and in-silico prediction tools, more insight into the actual scenario of NPS consumption was given.

In this thesis, 3 scientific articles were published within the framework of the international project co-funded by the European Commission: 'NPS-Euronet. Identification and assessment of new psychoactive substances: A European network' (HOME/2014/JDRUG/AG/DRUG/7086).

The first work (**scientific article 8**) reviews the different methodologies and approaches using LC-MS for monitoring NPS consumption through WW and PU analysis published within the period 2013-2018. PU samples collected at specific settings, *e.g.* music festival or nightlife clubs, together with WW analysis represent a valuable source of information to give light on the amount and number of NPS consumed. Additionally, in **scientific article 8** we give our point of view on the utility of PU and WW samples as well as the potential application of WBE as a source of information for NPS use.

The second work (**scientific article 9**) presents the development and validation of a methodology for the determination of 22 ID and NPS in wastewater by UHPLC-MS/MS. The compounds included in the study were based on NPS findings from previous studies within NPS-Euronet project published elsewhere (Salgueiro-González et al., 2019). Therefore, and due to the expected low concentration of the targets, a selective and sensitive methodology using QqQ was developed and applied to wastewater samples from different European locations. Several ID and NPS including dipentylone, butylone, mephedrone, methedrone and methylone were observed in WW samples, being dipentylone detected in WW for the first time. Additionally, a comprehensive study on the potential application of micro-liquid chromatography coupled to tandem mass spectrometry (μLC-MS/MS) for the determination of NPS in wastewater samples was included. Although an outstanding improvement in sensitivity was observed

(14-fold increase in average), the performance of μLC-MS/MS was not robust enough to permit a method validation since chromatographic retention was strongly affected by matrix composition.

Finally, the third work (**scientific article 10**) was completed in the form of a tutorial article presenting the main perspectives and hurdles faced during the determination of NPS. It gives a complete overview of analytical challenges such as complexity of matrices, sample preparation, high sensitivity and selectivity required in analytical methods as well as the rapid transience of NPS on the market. The **scientific article 10** also discusses the advantages and drawbacks of different acquisition workflows and data exploration approaches paying special attention to new developments such as ion mobility and *in-silico* prediction tools to improve the identification capabilities in complex matrices.

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

- *Current Opinion in Environmental Science & Health 9 (2019) 1-12*
- *Journal of Chromatography A 1602 (2019) 300-309*
- *Analytica Chimica Acta 1145 (2021) 132-147*

Chapter 5.2.1. Scientific Article 8

MONITORING NEW PSYCHOACTIVE SUBSTANCES USE THROUGH WASTEWATER ANALYSIS: CURRENT SITUATION, CHALLENGES AND LIMITATIONS

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Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations

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Keywords

New psychoactive substances; Pooled urine; Urban wastewater; Wastewater-based epidemiology; Mass spectrometry.

Abstract

New psychoactive substances (NPS) are compounds that produce similar effects to those induced by illicit drugs (IDs), such as cocaine, cannabis, and amphetamines, but are not strictly regulated by international conventions. The consumption of NPS is a growing public health problem in many communities. However, there is little knowledge regarding the extent and actual use of these new substances. Monitoring NPS use is arduous, and therefore, different sources of information need to be used to get more insight of the prevalence and diffusion of NPS use. Analysis of pooled urine and wastewater (WW) shows strong potential, giving a different and complementary light on this issue, although presents some limitations and challenges that must be taken into account. Liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) is one of the most powerful approaches for screening a large number of NPS because of the accurate-mass full-spectrum acquisition measurements. By using a comprehensive and updated NPS database, LC-HRMS is flexible enough to confront the ever-changing NPS market. In this "current opinion", we give our point of view on the usefulness of pooled urine and WW analysis and on the potential application of wastewater-based epidemiology as source of information for NPS use, explaining the main bottlenecks and future perspectives in this emerging research field.

1. Introduction

New psychoactive substances (NPS) can be defined as substances that produce similar effects to those induced by illicit drugs (IDs) such as cocaine, cannabis, and amphetamines but are not strictly regulated by international conventions [1]. Although many NPS are synthesized introducing only minor modifications to the chemical structures of controlled substances, the term 'new' does not directly refer to 'newly developed' chemicals but to 'newly misused' substances [2]. The NPS market is, therefore, very dynamic, creating, quickly, new alternative substitutes. Hence, the early warning system (EWS) of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) reported more than 670 different NPS between 2005 and 2017 [3]. NPS can be classified in different categories depending on their structural backbone. Cathinones and synthetic cannabinoids are most often reported, but also benzodiazepines, arylcyclohexylamines, phenethylamines, and synthetic opioids were found (**Figure 1**) [3]. These new drugs have become easily available to the general public mainly through e-commerce and are considered a growing problem in many communities because they are responsible for numerous fatal intoxications [4–6]. Although several countries have suffered the emergence of NPS, that is, use and harms, not all governments have been able to act upon all of them in an effective way in terms of penalizing their supply and use [7,8].

Understanding the extent and actual use of NPS is important for health-care professionals and toxicologists to assess the risks associated but also for policymakers to help orient prevention and define law enforcement activities. Different sources of information, such as general population surveys [9–11], EWS [3], internet [12], seizure data [13–16], and the analysis of biological samples (urine of users from hospital emergency rooms, postmortem fluids) [17–20], can be consulted to get insight into the prevalence and diffusion of NPS use.

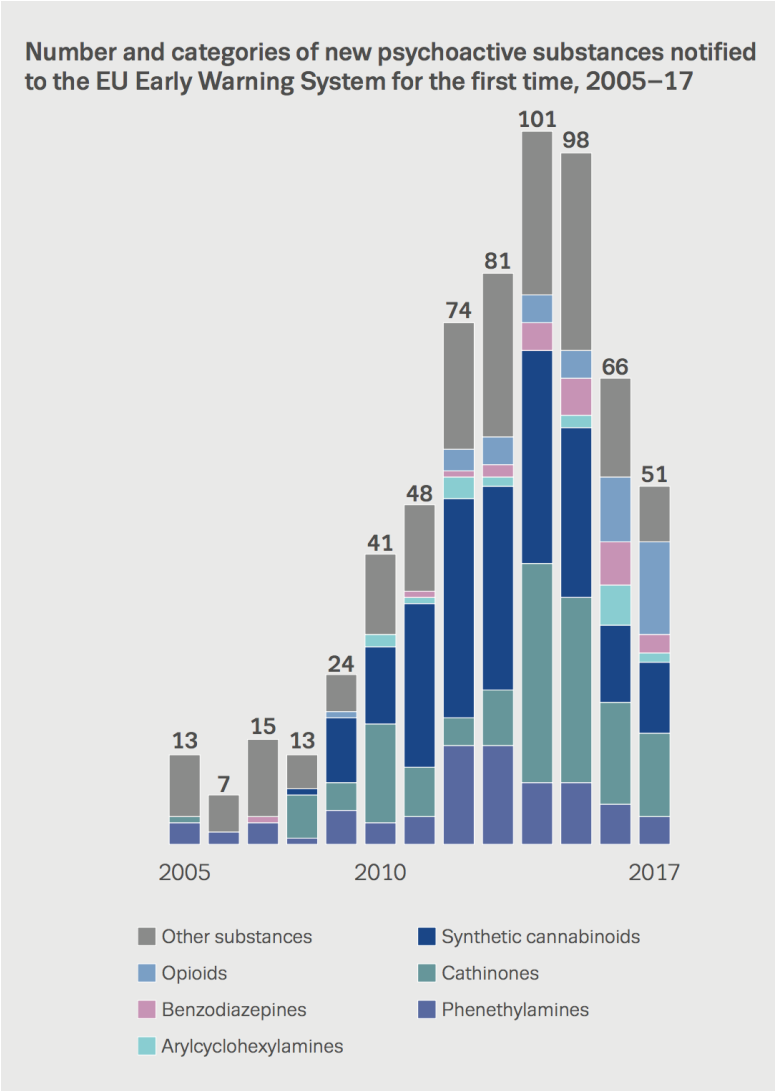

Figure 1. Number and categories of new psychoactive substances notified to the EU Early Warning System for the first time within 2005–2017 (reproduced with authorization from the European Drug Report 2018 of the EMCDDA [3]). EMCDDA, European Monitoring Centre for Drugs and Drug Addiction.

A recent approach that shed a different light on this issue is the analysis of pooled urine (PU) and urban wastewater (WW) samples. PU and WW analysis can provide anonymized but comprehensive and objective information on

community-wide use of NPS [21–24]. The wastewater-based epidemiology (WBE) approach relies on the fact that traces of almost everything humans consume are excreted, unaltered or as metabolites, via urine or feces [25]. Thus, the determination of appropriate urinary excretion products (biomarkers) and subsequent concentration data in WW can be used to estimate illicit and licit drug use by a population [25,26]. The Sewage Analysis CORe group Europe (SCORE) has promoted and coordinated WBE campaigns for the worldwide monitoring of ID consumption since 2011 [27–29], reporting the results to the EMCDDA, which considers WBE as a complementary source of information to the conventional indicators on drug use. In addition, the Australian Criminal Intelligence Commission (as part of their drug monitoring program: https://www.acic.gov.au/publications/intelligence-products/national-wastewater-drug-monitoring-program-report [30]) as well as New Zealand and China have set up strategies to implement such studies in their countries. For the proper application of WBE, however, several key aspects such as the selection of suitable and unique biomarkers and excretion rates need to be taken into account to obtain population-normalized quantitative data, that is, information on amounts consumed [31–34]. WBE has been successfully applied to the monitoring of tobacco [35,36], alcohol [37], and ID use [27,28,33,38] and has the potential to detect and discover newly consumed NPS [25,26,39,40].

In this review, we give our viewpoint on the monitoring of NPS in PU and WW and the potential application of WBE in this field, with special emphasis on challenges and limitations. Finally, future perspectives are briefly presented. The analysis of PU has been included in this article because of the very few studies available of NPS in WW (in comparison to conventional IDs) and the challenges to obtain information of NPS use from WW, as explained later in the manuscript. In addition, searching for NPS in PU can provide useful and complementary information on this topic.

2. Analytical challenges for monitoring NPS

The ever-changing nature of NPS poses a challenge for analytical forensic laboratories. The NPS market is very dynamic, and the rapid introduction of new substances makes it highly difficult to keep the analytical methodologies up to date. The detection, identification, and quantification of NPS is time consuming, complex, and expensive. However, identifying the new substances that are appearing in the market is the first necessary step in assessing the risks associated with these substances and in controlling potentially dangerous new drugs. Under these circumstances, the analysis of commercially available products (sometimes known as 'legal highs') provides updated information of the compounds possibly consumed. A combination of several techniques, such as nuclear magnetic resonance (NMR), high-resolution mass spectrometry (HRMS), gas chromatography mass spectrometry (GC–MS), X-ray crystallography, Fourier transform infrared (FTIR) spectrometer, and ultraviolet and circular dichroism, is often needed for a full characterization and true confirmation of the identity of unknown new drugs [41–46].

The continuous appearance of new substances joined to the limited availability of reference standards and difficulties to purchase them make the development of quantitative target methods somehow a limited approach and nonaffordable task when monitoring hundreds of changing NPS. Therefore, there is an increasing interest in developing qualitative screening methodologies able to detect and identify a large number of compounds. The hyphenation of liquid chromatography (LC) with HRMS is one of the most powerful approaches to this aim [47–49]. LC-HRMS appears as the technique of choice due to the polar character of most NPS, especially of metabolites, and the useful information contained in accurate-mass full-spectrum acquisition data. The main reason for the shift toward qualitative, suspect screening methodologies based on LC-HRMS is that there is, in principle, no need of reference standards for tentative identifications and the list of compounds that can be searched is only limited by the suspect screening database [39,47,48,50,51]. To help in the identification of NPS, a new web-based database (NPS Data Hub) has been developed with the aim to elicit data from the forensic laboratories to facilitate identification of

unknown substances [52]. In this way, the time for valuable data to be accessible to analytical laboratories for identification of newly emerging compounds is notably reduced. Analytical data of any type can be added for a given compound, but the mostly applied techniques are NMR, (HR)MS, and infrared/Raman. The combination of a compound database and HRMS spectral library represents a useful tool for the identification of NPS in forensic HRMS-based screening applications [53].

If the identification of NPS in commercially available products (herbal blends, powder, pilots, crystals, etc.) is complex, the detection and identification of NPS residues in urine samples is even more challenging. Unfortunately, most of the aforementioned techniques are not useful in this type of analysis because of the low analyte concentrations in the samples and the complex nature of the urine matrix with endogenous components being at concentrations much higher than those of the NPS potentially consumed. In addition, the low rate of positive findings when analyzing individual urine samples complicates even more the monitoring of NPS. To this aim, the analysis of PU samples from hundreds (or thousands) of individuals at specific settings with higher probability of NPS consumption is preferred. Nightlife areas, music festivals, or local festivities are strategic locations for the collection of PU samples from the inner container of pissoirs or portable toilets. The likelihood of having NPS consumers among all PU contributors increases the rate of success in identifying NPS consumed.

Additional difficulties appear in the investigation of NPS in WW, mainly because of the extremely low concentrations of NPS due to the lower consumption in comparison with popular, conventional IDs and to the high dilution factor in WW. The main drawback of LC-HRMS screening of NPS in PU or WW comes from its lower sensitivity compared with target quantitative methods (*e.g.* by LC-MS/MS with triple quadrupole [QqQ]), an aspect that is crucial in this field. In addition, strong ionization suppression commonly occurs on the analyte signal in these complex matrices. For this reason, the target quantitative methods (*e.g.* LC-MS/MS QqQ) are still valuable, although they are restricted to the limited target list of compounds included in the scope of the method, with the

corresponding reference standards being required for method optimization, data acquisition, and quantification [23,24,49,54–57].

Another relevant issue is NPS metabolism, which plays a key role for the selection of appropriate biomarkers (parent compound or metabolites) for monitoring NPS in PU or WW. Owing to the general lack of information on metabolic pathways for many NPS, there is a great interest in the scientific community to perform metabolic studies to identify compounds proposed as target compounds in urine or in WW [58–62]. However, even if information of the major metabolites is available from the literature, their analysis can be complicated due to the lack of reference standards, and therefore, only tentative identifications may be possible using HRMS.

3. Investigation of NPS in PU

The analysis of urine from intoxication cases or potential consumers seems, a priori, a suitable source of information for the monitoring of NPS [5,49]. However, it is not easy to obtain these samples, and the consent of the users or family members is required. The analysis of PU collected from places with higher probability of NPS consumption (*e.g.* discotheques, music festivals, or nightlife areas) can give a more realistic picture of the NPS situation within a population. Besides, samples are anonymized, and ethical issues are limited [63,64].

Table 1 summarizes the studies on PU analysis for NPS reported in the last five years. The vast majority of these studies applied the potential of LC-HRMS for qualitative identification of NPS using time-of-flight [48,65–67] or Orbitrap [68] mass analyzers. A few studies focused on a limited list of target compounds, which were quantified using low-resolution mass analyzers (LC-MS/MS QqQ) [21].

The selection of specific settings for the analysis of PU increases the degree of success in the detection of NPS. For this reason, 60% of the studies reported data from music festivals because of the higher probability of drug or NPS consumption [48,65–67]. Samples were collected from urine containers of pissoirs or from portable toilets, resulting in an anonymous mixture of urines from an undetermined numbers of contributors. It is remarkable that most studies collected samples from pissoirs, resulting in cleaner samples than those collected in portable toilets. The latter are contaminated with feces and disinfection chemicals, which may have an unknown effect on NPS stability. Furthermore, it must be taken into account that pissoirs are designed for men, and thus only represent a part of the setting.

In these works, the most commonly detected NPS categories were synthetic cathinones and phenethylamines. It seems logical that mainly invigorating drugs were found because music festivals and nightlife locations are more prone to the intake of stimulant compounds. Paying attention to the individual NPS consumed, mephedrone [21,66,68] and ketamine [22,66–68] were the most reported drugs in PU analysis.

Table 1. Summary of recently reported studies on NPS determination in Pooled Urine samples.

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
United Kingdom (City of Westminster, London)	Pooled urine Weekend sampling Pissoir (male urinal)	1700 compounds (ID, NPS and *metabolites*)	ketamine, hordenine, d-norpseudoephedrine, methylhexanamine, 4-methylmethcathinone, methopropamine and *metabolites*, methoxetamine and *metabolites*	SPE, LLE LC-MS/MS [b] Qualitative	Archer, 2013 [22]
Norway (Oslo)	Pooled urine Sampling during festival Pissoir (male urinal)	ID, NPS	hordenine, 1-(2-methoxyphenylpiperazine), cathinone	UHPLC-QTOF Qualitative	Reid, 2014 [65]
United Kingdom (Night Club in London)	Pooled urine Weekend sampling Pissoir (male urinal)	900 compounds (ID, pharmaceuticals, steroids, NPS and *metabolites*)	mephedrone and *metabolites*, TFMPP and *metabolites*, 2-aminoindane	SPE, LLE, shoot techniques LC- MS/MS [b] Qualitative and quantitative	Archer, 2014 [21]
United Kingdom (City of Westminster, London)	Pooled urine Weekend sampling Pissoir (male urinal)	ID, NPS	mephedrone, methylhexaneamine, methiopropamine, pipradol, cathinone, 5-APB, 4-methylethcathinone, TFMPP, 4-methylbuphedrone, methcathinone, ethylmethcathinone, d-norpseudoephedrine, ketamine, 1,4-methoxyphenylpiperazine, 4-fluoroephedrine	SPE UHPLC-LTQ Orbitrap Qualitative	Archer, 2014 [68]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
United Kingdom (City center and festival) and Belgium (festival)	Pooled urine Weekend sampling in the city and during festivals Pissoir (male urinal)	1500 compounds (ID, NPS and *metabolites*)	MPA, methylone, ethylone, methedrone, mephedrone, *dyhidromephedrone*, *normephedrone*, 5-APB, ketamine, *norketamine*, *hydroxynorketamine*, *dehydronorketamine*, 4-FA, α-PVP, *M-264 and M-234 (α-PVP metabolites)*, hordenine, methoxetamine	UHPLC-QTOF Qualitative	Kinyua, 2016 [66]
Norway (Festivals)	Pooled urine Sampling during festivals Pissoir and portable toilets	Suspect screening: 1000 compounds (including ID, pharmaceuticals and 16 NPS) Target: 51 compounds (including synthetic cathinones, phenethylamines, ketamine and phencyclidine-type sustances)	methylphenidate, BZP	SPE UHPLC-QTOF Qualitative	Baz-Lomba, 2016 [48]
Denmark (Festival, Roskilde)	Pooled urine Sampling during festival Portable toilets	467 compounds (ID, NPS and *metabolites*)	ketamine, methylphenidate	SPE UHPLC-QTOF Qualitative	Hoegberg, 2018 [67]

NPS, new psychoactive substance; **ID**, illicit drug; **4-chloro-α-PPP**, 4'-chloro-α-pyrrolidinopropiophenone; **4-FA**, 4-fluoroamphetamine; **5-APB**, 5-(2-aminopropyl)benzofuran; **α-PVP**, α-pyrrolidinovalerophenone; **BZP**, 1-benzylpiperazine; **M-234,** 1-phenyl-2-(pyrrolidin-1-yl)pentan-1-ol; **M-264**, hydroxy-4-((1-oxo-1-phenylpentan-2-yl)amino)butanal; **MPA,** methiopropamine**; TFMPP**, trifluoromethylphenylpiperazine; **SPE**, solid-phase extraction **LC**, liquid chromatography; **MS**, mass spectrometry; **LLE**, liquid-liquid extraction; **UHPLC**, ultra-high performance liquid chromatography; **LTQ**, linear ion trap; **QTOF**, quadrupole time-of-flight.
[a] NPS metabolites highlighted in italic letters. [b] No information available about the specified analytical technique used

4. Investigation of NPS in WW

The application of WBE for the estimation of psychoactive substance consumption is mainly focused on ID [25] and has been scarcely applied to NPS. As mentioned in previous sections, the investigation of NPS in WW is very complicated due to several factors that make the full application of WBE to NPS still quite limited. The lack of information on excretion rates and metabolic pathways of NPS and the very low concentrations in WW are the main drawbacks. The majority of the published studies on NPS in WW only dealt with detections and concentrations, without producing either mass loads (*i.e.* concentrations multiplied by flow rates of WW) or normalized data to the population within the WW catchment area.

Table 2 summarizes the main developments in the monitoring of NPS consumption through WW analysis. The vast majority of reported studies applied solid-phase extraction for the preconcentration of target compounds followed by LC-MS/MS (*QqQ*) analysis because of the enhanced sensitivity of this type of mass analyzers [23,24,49,54–57,69–77]. However, there are also studies using LC-HRMS [47–49,51,76,78–82]. Although back calculations to estimate the consumption of NPS by a population is complicated and, for now, unrealistic, the quantification of NPS (as in most LC-MS/MS methods) may give a better comprehension of the actual use when comparing with the mass loads found for conventional IDs.

Several studies focus the collection of samples on weekends, festivities, or festivals, when higher concentrations of NPS in WW are expected [24,47,56]. In general, 24-h composite samples are collected at the entrance of a WW treatment plant.

The NPS most found in WW are synthetic cathinones. Thus, 21 of the 30 reviewed studies reported positive findings of at least one synthetic cathinone; of which, methylone [23,48,49,54,55,74,78,79,83–85] and mephedrone [23,24,49,54,70,72–74,79,81,85] were most often reported. Despite the fact that these compounds are currently illegal in many countries, they seem to be well established in the drug market, showing a recurrent detection in WW. Other NPS,

such as synthetic cannabinoids, were scarcely detected [47,56,57,78,84], which could be related to the fact that synthetic cannabinoids are highly and quickly metabolized by humans [86,87], and therefore should be mostly found as major metabolites in WW. The particular case of synthetic opioids is of major concern because of the epidemic increase of opioids consumption over the last years, especially in the US [88], with alarming news stories in the ordinary press [89–91]. Recently, the first detection of fentanyl and metabolites was reported by different studies in Europe and the US [69,74,92].

Some compounds included in **Table 1** and **Table 2** might not be considered as NPS as it is very difficult to differentiate these compounds being used illicitly or legally. For example, hordenine is present in beer but some studies considered this substance as a 'potential NPS' [22,47,65,66]. Also, ketamine is used for certain applications as veterinary and medical drug but is considered as a recreational substance by the EMCDDA. Besides, as stated previously, this organism defines NPS as 'newly misused' substances, which embraces these cases of chemicals intended for other purposes than for which it is originally developed.

The most of the scientific production about determination of NPS in WW is performed over 2016 [48,51,73,74,81–83], 2017 [23,47,75,79,80,84], and 2018 [49,69–71,76,77,92,93], with Europe being the most productive region [23,24,47,48,51,54-57,69,71-73,77,79-82,93], followed by Australia [49,74,76,78,83,85]. Asia [75,94], US [92], and Africa [70] have barely applied strategies for NPS monitoring through WW analysis.

Table 2. Summary of recently reported studies on NPS determination in WW samples.

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
Australia (Adelaide)	24 h composite	MDMA, methcathinone, mephedrone, methylone, MDPV, BZP, TFMPP	methcathinone, mephedrone, methylone, MDPV, BZP, TFMPP	SPE UHPLC-Qtrap Quantitative	Chen, 2013 [85]
Norway (Oslo, Bergen, Harmar)	72 h composite Weekend sampling	14 NPS (synthetic cathinones, *metabolites* of synthetic cannabinoids and phenethylamines)	d-norpseudoephedrine, pseudoephedrine, *JWH-018 N-5-hydroxypentyl*	SPE UHPLC-QqQ Quantitative	Reid, 2014 [56]
Belgium (Antwerp, Boechout, Ninove, Ruisbroek, Zele) and Switzerland (Zurich)	24 h composite	methoxetamine, butylone, ethylone, methylone, MPA, PMMA, PMA	methoxetamine, butylone, ethylone, methylone, PMMA	SPE LC-QqQ Quantitative	Kinyua, 2015 [55]
South Korea (Busan, Ulsan, Changwon, Kimhae, Milyang)	24 h composite	17 compounds (ID, ketamine, *norketamine*, mephedrone and methylone)	None	SPE UHPLC-Qtrap Quantitative	Kim, 2015 [94]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
Greece (Santorini Island)	Grab	10 NPS (synthetic cannabinoids, cathinones, piperazines and pyrrolidophenones)	JWH-210, JWH-122, α-PVP, CP47,497	SPE UHPLC-QqQ Quantitative	Borova, 2015 [57]
Croatia (Zagreb, Vinkovci, Velika Gorika)	24 h composite and grab	25 NPS (mainly synthetic cathinones and other substituted phenylalkylamines)	flephedrone, methylone, methedrone, mephedrone, ketamine, *norketamine*	SPE LC-QqQ Quantitative	Senta, 2015 [54]
Italy (from 17 cities)	24 h composite	ketamine, mephedrone	ketamine, mephedrone	SPE UHPLC-QqQ Quantitative	Castiglioni, 2015 [72]
Spain (Valencia)	24 h composite	Target: 42 compounds (21 emerging psychoactive substances) Suspect screening: 2000 compounds (pharmaceuticals, pesticides, mycotoxins and psychoactive substances)	Target: ephedrine Suspect screening: ephedrine, ethylamphetamine, α-PVP,4'MePHP, ketamine, *methylephedrone*	SPE UHPLC-QTOF MS/MS Quantitative and qualitative	Andrés-Costa, 2016 [82]
Italy (Milan, Bologna, Turin, Perugia)	24 h composite	52 NPS (synthetic cannabinoids, synthetic cathinones, ketamine derivatives, phenethylamines and others)	None	SPE UHPLC-LTQ Orbitrap Qualitative	González-Mariño, 2016 [51]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
United Kingdom (Bath)	24 h composite	56 compounds (ID, pharmaceuticals, mephedrone, ketamine, benzylpiperazine, ephedrine, pseudoephedrine and PMA)	mephedrone, ketamine, benzylpiperazine, ephedrine	SPE UHPLC-QqQ Quantitative	Castrignano, 2016 [73]
Italy (Florence, Bologna, Turin, Perugia, Milan), Spain (Santiago de Compostela), Norway (Oslo) and United Kingdom (Southwest)	24 h composite Weekend sampling	18 synthetic cathinones	mephedrone, N,N-dimethylcathinone, methcathinone, 4-FMC, 4-MEC, MDPV, ethylone	SPE UHPLC-QqQ Quantitative	González-Mariño, 2016 [24]
Australia (South East Queensland)	24 h composite	methylone, mephredone	methylone	Direct injection LC-QqQ Quantitative	Thai, 2016 [83]
Poland (Plaszow, Krakow)	24 h composite	MDMA, mephedrone, 4-MEC, MDPV, mCPP	mephedrone, 4-MEC	SPE LC-QTOF Quantitative	Styszko, 2016 [81]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
Australia (Adelaide)	24 h composite	21 compounds (ID and 10 NPS)	methylone, methcathinone, MDPV, BZP, mephedrone, TFMPP, α-PVP	SPE LC-QqQ Quantitative	Tscharke, 2016 [74]
Norway (Oslo, Trondheim)	24 h composite	51 compounds (ID, pharmaceuticals and 16 NPS)	methylone, ketamine, methoxetamine	SPE (POCIS) UHPLC-QTOF Qualitative	Baz-Lomba, 2016 [48]
The Netherlands (Amsterdam)	24 h composite Sampling during festival	2000 compounds (including ID, pharmaceuticals and NPS)	PMMA, methylhexanamine, 4-fluoroamphetamine, MDEA, mCPP, 2C-B, fentanyl, L-759,633, ketamine, hordenine	SPE UHPLC-QTOF UHPLC-LTQ Orbitrap Qualitative	Causanilles, 2017 [47]
European cities (Zurich, Copenhagen, Oslo, Castellon, Milan, Brussels, Utrecht, Bristol)	24 h composite	10 NPS (cathinones and phenethylamines)	MDPV, mephedrone, methylone	SPE UHPLC-QqQ Quantitative	Bade, 2017 [23]
Spain (Tarragona, Reus)	24 h composite	10 compounds (ID, mephedrone, 4-methylephedrine and MDPV)	None	SPE UHPLC-Exactive Orbitrap Quantitative	Prosen, 2017 [80]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
New Zeeland (Auckland)	24 h composite	17 compounds (ID, methylone, ketamine *norketamine*, mephedrone, JWH-073 and JWH-018)	methylone, JWH-018	Direct injection, SPE LC-QqQ Quantitative	Lai, 2017[84]
China (18 major cities)	24 h composite	Mephedrone, MDPV, BZP, TFMPP, mCPP	MDPV, BZP	SPE UHPLC-QqQ Quantitative	Gao, 2017 [75]
Spain (Tarragona)	24 h composite	12 synthetic cathinones and one metabolite	flephredone, methylone, buphedrone, 4-methylephedrine, butylone, mephedrone, pentedrone, 3,4-DMMC, α-PVP, MDPV	SPE UHPLC-Exactive Orbitrap Quantitative	Fontanals, 2017 [79]
South Australia	24 h composite	Qualitative: 346 compounds (ID, pharmaceuticals and NPS) Target: subset of these compounds	α-PVP, MDPV	SPE UHPLC-QqQ UHPLC-QTOF Quantitative and qualitative	Bade, 2018 [76]
South Australia	24 h composite	187 NPS	Qualitative: α-PVP, ethylone, MDPV, mephedrone, methcathinone, methylone, BZP, TFMPP, pentylone, 25H-NBOMe, MDA Quantitative: butylone, ethylone, α-PVP, methcathinone, MDPV, pentylone, mephedrone	SPE UHPLC-QqQ UHPLC-QTOF Quantitative and qualitative	Bade, 2018 [49]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
Spain (Santiago de Compostela)	24 h composite	38 compounds (ID, pharmaceuticals, mephedrone, ketamine and mCPP)	None	SPE UHPLC-QqQ Quantitative	González-Mariño, 2018 [77]
Norway (Trondheim)	24 h composite	8 compounds (THC, 3 metabolites of THC and 4 metabolites of synthetic cannabinoids)	None	LLE UHPSFC-QqQ Quantitative	González-Mariño, 2018 [93]
Croatia (Zagreb, Split)	24 h composite	27 opioids and metabolites	Detection of fentanyl, *norfentanyl* and sufentanil	SPE UHPLC-QqQ Quantitative	Krizman-Matasic, 2018 [69]
USA (Southwestern university campus)	24 h composite	19 compounds (ID and metabolites, oxycodone, fentanyl, buprenorphine, methylphenidate, alprazolam)	fentanyl, *norfentanyl*	Isotope dilution (ID-LC-MS/MS) Quantitative	Gushgari, 2018 [92]
South Africa (Johannesburg, Cape Town)	24 h composite	18 compounds (ID, mephedrone, ephedrine, pseudoephedrine, *norephedrine*)	mephedrone	SPE UHPLC-QqQ Quantitative	Archer, 2018 [70]

Sampling area	Type of sample	Compounds [a]	NPS positive findings [a]	Analytical technique	Reference
Spain (Barcelona)	24 h composite	37 compounds (ID, pharmaceuticals, ephedrine, mephedrone, ketamine, methoxetamine, MDPV)	None	On-line SPE UHPLC-QqQ Quantitative	López-García, 2018 [71]
Australia	24 h composite and grab	187 NPS	Confirmed: MDA, AM-2201, UR-144, 4-FMC, α-PVP, ethylone, methcathinone, methylone, pentedrone, methoxetamine Detected: 5F-APINACA, JWH-018, JWH-073, 4-MEC, butylone, mephedrone, pentylone, U-47700, methiopropamine	SPE UHPLC-QTOF Qualitative	Bade, 2019 [78]

NPS, new psychoactive substance; **ID**, illicit drug; **2C-B**, 4-bromo-2,5-dimethoxyphenethylamine; **25H-NBOMe**, 2,5-dimethoxyphenethylamine; **3,4-DMMC**, 3,4-dimethylmethylcathinone; **4-FMC**, 4-fluoromehcathinone; **4-MEC**, 4-methylcathinone; **4'MePHP**, 4′ -methyl-α-pyrrolidinohexanophenone; **5F-APINACA**, N-(adamantan-1-yl)-1-(5-fluoropentyl)-1H-indazole-3-carboxamide; **α-PVP**, α-pyrrolidinovalerophenone; **MA-2201**, 1-(5-fluoropentyl)-3-(naphthalen-1-oyl)indole; **BZP**, 1-benzylpiperazine; **CP47,497**, 2-[(1S,3R)-3-Hydroxycyclohexyl]-5-(2-me thyl-2-octanyl) phenol; **JWH-018**, 1-Naphthyl (1-pentyl-1H- indol-3-yl) methanone; **JWH-073**, 1-naphthyl (1-butyl-1H-indol-3-yl) methanone; **JWH-122**, 4-Methyl-1-naphthyl) (1-pentyl-1H-indol-3-yl) methanone; **JWH-210**, (4-Ethyl-1-naphthyl)(1-pentyl-1H-indol-3-yl) methanone; **L-759,633**, (6aR,10aR)-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-methoxy-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; **mCPP**, 1-(3-chlorophenyl)piperazine; **MDA**, 3,4-methylenedioxyamphetamine; **MDEA**, 3,4-methylenedioxyethylamphetamine; **MDPV**, methylenedioxypyrovalerone; **MPA**, methiopropamine; **PMA**, 4-methoxyamphetamine; **PMMA**, 4-methoxymethamphetanime; **TFMPP**, trifluoromethylphenylpiperazine; **U-47700**, 3,4-Dichloro-N-[(1R,2R)-2-(dimethylamino)cyclohexyl]-N-methylbenzamide; **UR-144**, (1-pentylindol-3-yl)-(2,2,3,3-tetramet hylcyclopropyl)methanone; **SPE**, solid-phase extraction; **LC**, liquid chromatography; **QqQ**, triple quadrupole; **MS**, mass spectrometry; **POCIS**, passive organic chemical integrative samplers.
[a] NPS metabolites highlighted in italic letters.

5. Future perspectives

Monitoring NPS use through PU and WW analysis is a challenge due to several factors: *(1)* their rapid transience on the drug market creates a scenario with constantly moving analytical targets; *(2)* the lack of data on NPS metabolism and pharmacokinetics, that is, for the selection of unique biomarkers and information on excretion rates; *(3)* the lack of data on stability of potential biomakers in urine and sewage; *(4)* the generally very low concentrations, because of the high choice for consumers in number of compounds, the low dose of some NPS and low prevalence in use, plus the elevated dilution factor of WW, that is, dilution of urine and feces with water used in households, industry, and so forth; and *(5)* the high sensitivity and selectivity required in the analytical methods, as a consequence of the low analyte concentrations and the complexity of the sample matrix.

Target quantitative methods based on LC-MS/MS QqQ, although limited by the target list of compounds, are useful because of the excellent sensitivity of this technique. However, LC-HRMS is the technique of choice for screening a large number of both NPS and metabolites. Hence, the maintenance of comprehensive and updated databases is essential. Data from surveys, police seizures, forensic analyses, as well as from EWS, and the scientific literature are necessary. The database should be fed with information from analysis of the products potentially consumed (*e.g.* herbal blends, crystals, pilots, powder purchased online or from smart shops), where nontargeted analytical strategies may be necessary to identify unexpected or unknown compounds, to include substances that are actually sold on the market. Furthermore, the inclusion of metabolites in the database is pivotal for realistic studies as it will allow focusing the analysis on those targets that are more likely present in urine and WW samples.

Figure 2 illustrates the different steps and topics that should be considered to get a comprehensive overview on NPS use, including analysis of WW and PU as one of the key issues.

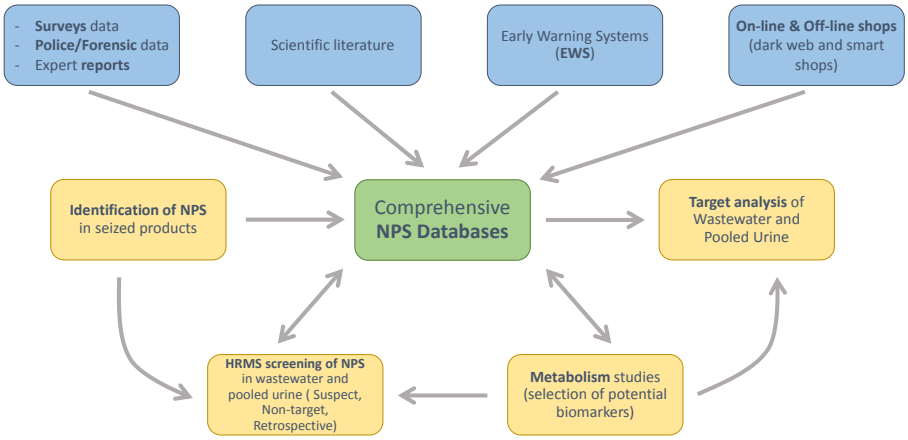

Figure 2. Sources of information, steps, and topics required to build a comprehensive database for monitoring NPS use. NPS, new psychoactive substance; HRMS, high-resolution mass spectrometry.

As can be seen, the scenario around NPS use is rather complex. Lot of research is required in the next years to provide more information in different areas, with analytical chemistry playing a key role. Close collaboration is needed between different disciplines and actors that are relevant in the drugs scenario. This scenario includes not only collaboration between analytical chemists but also toxicologists, health professionals, as well as police forces, national governments, national focal points, and organizations such as EMCDDA and United Nations Office on Drugs and Crime (UNODC).

Regarding WW analysis, more information is required for full application of WBE, such as excretion rates and stability of NPS in sewage, to obtain estimates of NPS consumed. Despite the limitations, data from screening WW (and PU) is highly valuable to understand the extent and actual use of NPS within certain populations, at least of those most widely consumed. In this context, HRMS screening of WW and PU collected from special settings (*e.g.* in festivals, near discotheques, or nightclubs), where higher NPS consumption is expected, is a good strategy. The possibility to re-evaluate HRMS data in a retrospective way, without the need of additional analysis, is worth noticing as it allows re-

examining data previously obtained, searching for new/additional compounds not considered in the initial analysis.

As illustrated in the workflow of **Figure 2**, different sources are needed to get a broad overview of NPS use. Data triangulation, that is, combining information obtained from PU and WW analysis with other sources, such as survey data and forensic data, seems one of the best approaches nowadays to get a comprehensive insight into the NPS situation [49].

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Chapter 5.2.2. Scientific Article 9

SIMULTANEOUS DETERMINATION OF NEW PSYCHOACTIVE SUBSTANCES AND ILLICIT DRUGS IN SEWAGE: POTENTIAL OF MICRO-LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY IN WASTEWATER-BASED EPIDEMIOLOGY

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Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology

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Highlights

- Simultaneous monitoring of 5 illicit drugs and 17 NPS in wastewater.
- Low sample and organic solvent volumes used resulted in a greener methodology.
- Micro-LC was extensively evaluated in terms of sensitivity and reproducibility.
- Micro-LC has shown a strong potential for wastewater-based epidemiology application.
- Dipentylone has been found for the first time in wastewater.

Keywords

Capillary liquid chromatography; New psychoactive substances; Wastewater analysis; Drugs of abuse; Tandem mass spectrometry; ionKey.

Abstract

Wastewater-based epidemiology (WBE) can give valuable light on the extent and actual use of new psychoactive substances (NPS). In this work, a fully validated methodology for the simultaneous determination of illicit drugs and NPS in wastewater by solid-phase extraction followed by UHPLC-MS/MS has been developed. The small sample volume (5 mL) required for analysis is of high interest, especially when performing large sampling campaigns involving many locations of different geographical origin, as it has been performed in the past.

The method was applied to wastewater samples from different European locations and permitted the simultaneous monitoring of conventional drugs and NPS. Cocaine, amphetamine, MDMA, methamphetamine and ketamine were found in all wastewater samples, and several NPS (dipentylone, butylone, mephedrone, methedrone and methylone) were observed in some of the samples monitored. It is noteworthy that dipentylone was detected in wastewater for the very first time. Furthermore, a detailed comparison of micro liquid chromatography (μLC) and UHPLC, both coupled to tandem mass spectrometry, in terms of sensitivity and reproducibility has been made for the first time in the application field of WBE. An average increase factor of 14 (mass normalized data) was observed in sensitivity for μLC-MS/MS. The overall method performance was also compared (un-normalized data), and an average increase sensitivity factor of 4.5 was observed for μLC-MS/MS. However, large deviations in retention time (up to 0.4 min) affected the reproducibility and robustness of the μLC-MS/MS method when it was applied to wastewater analysis. Although in this work μLC-MS/MS was strongly influenced by the amount of matrix loaded in the separation device, its enhanced sensitivity and promotion of green chemistry (faster analysis time and less solvent consumption) allow to expect improved future applications, especially when analytes are present at very low concentrations.

1. Introduction

The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) has recorded more than 670 new psychoactive substances (NPS) comprising mainly synthetic cathinones, synthetic cannabinoids and phenethylamines [1]. The easiness on modifying the chemical structure of a psychoactive substance to produce a new one to avoid regulation and maintain clandestinity has contributed to the development of such large number of available NPS [2]. Therefore, the identification and quantification of NPS in different matrices of interest is a high-demanding analytical challenge [3,4].

Several approaches have been reported for the determination of NPS through the analysis of urine, post-mortem fluids, hair, wastewater (WW) or seized products [5–11]. WW seems a suitable matrix for the determination of NPS since it provides an overview of what is being consumed in a whole community. Consumed drugs are excreted as either parent compound or metabolites and end up in the sewage system, finally entering the wastewater treatment plants (WWTPs). By collecting a representative sample of influent WW, a huge anonymous urine test of a certain community can be obtained. Some analytical approaches use high resolution mass spectrometry (HRMS) for the analysis of NPS in WW [7,10–14] based on the strong identification potential of this technique. However, the sensitivity cannot be sufficient to detect and identify these compounds present at very low concentration levels in these complex matrices [13]. LC coupled to tandem mass spectrometry (UHPLC-MS/MS) with triple quadrupole (QqQ) appears as one of the most convenient tools to this aim, as it allows to quantify very low analyte concentrations. This technique has been in fact applied for the determination of some NPS in WW, albeit only a small amount of NPS have been detected [5–7,15].

Recently developed micro fluidic chromatographic techniques, such as micro-liquid chromatography (μLC) coupled to tandem mass spectrometry (μLC-MS/MS) allow to reach limits of detection and quantification lower than conventional UHPLC-MS/MS [16]. Reduction of internal diameter of the chromatographic column and flow rate at μL min^{-1} level are responsible for this

enhanced sensitivity [17,18]. By the utilization of low flow rates (<100 μL min^{-1}) the ionization efficiency is higher. In addition, electrospray plume reduces in size and, therefore, sampling efficiency in the electrospray source is greatly improved [19]. Since low volumes are involved in this equipment, special attention needs to be paid to dead volumes in connections and injection valve. Newly developed integrated μLC-ESI chip MS interfaces greatly reduce the problems related to dead volumes in laboratory-assembled μLC equipment [17,18,20]. Short analysis time, lower mobile phase consumption, higher sensitivity, lower sample volume and easy coupling to mass spectrometry are some of the main advantages of μLC [18,20]. Therefore, μLC may be a valuable technique for the development of green analytical methodologies because of the reduced usage of organic solvents and reduced sample volume. However, analysis time is often larger than in convention UHPLC and, due to the smaller dimensions, sample composition may compromise the μLC separations [16,18]. These two aspects are relevant drawbacks that need to be fully addressed during method optimization and sample analysis.

μLC-MS/MS has been applied in food [18,21], urine [17,22], plasma [23,24], serum [16] and blood [25] matrices for the determination of pharmaceuticals [16,17,22,24,25], pesticides, toxins and organic contaminants [18], polyphenols [21] and proteins [23]. However, we have not found previous applications of μLC-MS/MS to the analysis of NPS and illicit drugs (ID) in WW matrices.

In this work, a sensitive method for the simultaneous determination of 22 compounds, including NPS, ID and some main metabolites, by means of UHPLC-MS/MS (QqQ) has been developed and validated. The methodology applied minimizes the amount of sample necessary for analysis allowing to simplify sampling and delivering expenses, which is of relevance in large campaigns involving several WWTPs from different geographical locations. The NPS included in the method were selected based on their possible use as a replacement of conventional recreational ID, and are among those mostly reported. In addition, the potential application of micro-liquid chromatography for the determination of NPS in WW has been evaluated, with a detailed discussion of the advantages and drawbacks of this approach. A thoroughly

comparison of μLC–MS/MS and UHPLC-MS/MS has been made in terms of sensitivity and method reproducibility. This is, to the best of our knowledge, the first contribution where the potential of μLC-MS/MS has been evaluated for the monitoring of ID and NPS in WW.

2. Experimental

2.1. Chemicals and materials

In total, 22 ID, NPS and main metabolites were included in this study. Further details about the chemicals and materials used in this study can be consulted in **Supporting Information**.

2.2. Sample treatment

Before being extracted by solid-phase extraction (SPE), raw WW was centrifuged at 6000 rpm for 5 min. Then, 5 mL aliquot was spiked with 40 µL of 50 µg L^{-1} solution of isotope-labelled internal standards (ILIS), resulting in concentration of 400 ng L^{-1} in raw WW. After conditioning SPE cartridges with 2 × 3 mL of MeOH and 2 × 3 mL of H_2O, WW samples were extracted by means of Oasis HLB (60 mg, 3 cm^3) SPE cartridges. After loading the sample, cartridges were rinsed with 50 mL of Milli-Q water to reduce the amount of matrix interferences extracted in the SPE. The retained compounds were eluted with 1 mL of MeOH that was then evaporated at 40 °C under vacuum conditions for around 90 min in a miVac DUO Concentrator (Genevac, Italy). Extracts were reconstituted with 200 µL of H_2O:MeOH (90:10) and filtered through 0.22 µm × 4 mm nylon filters (Membrane Solutions, Plano, TX, USA). With this procedure, the preconcentration factor was 25. Finally, 3 µL of filtered extract was injected in the UHPLC-MS/MS system. Evaluation and optimization of SPE, centrifugation and evaporation steps can be found in **Supporting Information**. A summary of the sample procedure is presented in **Figure S1**.

2.3. Instrumentation

2.3.1. UHPLC-MS/MS analysis

UHPLC-MS/MS sample analysis was performed using a Waters Acquity H-class UPLC system (Waters Corporation, MA, USA) coupled to a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK) equipped with an electrospray ionization source (ESI) operated in positive ionization mode. Chromatographic separation was performed by means of an Acquity UPLC BEH C18 column (1.7 µm, 50 × 2.1 mm) from Waters at a flow rate of

300 µl min^{-1}. Column temperature was kept at 40 °C and sample manager was kept at 7 °C. Elution was performed with gradient of A: H_2O 0.01% HCOOH and B: MeOH 0.01% HCOOH, as follows: 0 min 10% B, 2 min 60% B, 2.50 min 90% B, 3.50 min 90% B, 3.60 min 10% B until 5.50 min for re-equilibrating the column for the next injection. Cone and desolvation gas were dry nitrogen set to 250 L h^{-1} and 1200 L h^{-1}, respectively. For the operation of MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) set to 0.15 mL min^{-1}. Source temperature was kept at 120 °C and capillary voltage was established at 3.0 kV. All data was acquired and processed using MassLynx v4.1 software (Waters, Manchester, UK).

2.3.2. µLC-MS/MS analysis

Sample analysis was performed using a Waters Acquity M-class UPLC system (Waters Corporation, MA, USA), equipped with a Peptide BEH C18 130 Å 1.7 µm 150 µm × 50 mm ionKey LC separation device (Waters Corporation) and interfaced to a triple quadrupole mass spectrometer (Xevo TQS, Waters Micromass, Manchester, UK) operated in positive ionization mode. iKey device was kept at 40 °C and sample manager was kept at 7 °C. Chromatographic separation was performed with gradient of A: H_2O 0.01% HCOOH and B: MeOH 0.01% HCOOH, as follows: 0 min 30% B, 2 min 60% B, 2.10 min 90% B, 3.10 min 90% B, 3.20 min 30% B until 5.50 min for re-equilibrating the column for next injection. Flow rate was established at 3 µL min^{-1}. Cone and nebulizer gas were dry nitrogen set to 250 L h^{-1} and 7 bar, respectively. For the operation of MS/MS mode, collision gas was argon 99.995% (Praxair, Madrid, Spain) set to 0.15 mL min^{-1}. Source temperature was kept at 120 °C and capillary voltage was established at 3.5 kV. All data was acquired and processed using MassLynx v4.1 software (Waters, Manchester, UK).

2.4. Quantitation

For quantitation purposes, data (both UHPLC-MS/MS and µLC–MS/MS) were acquired in selected reaction monitoring (SRM) mode. Three MS/MS transitions were acquired for each compound, using the most intense or selective one as the quantitation transition, and the other two transitions for confirmation purposes.

All compounds were quantified using its corresponding ILIS as surrogate internal standard. If no ILIS was available, an analogue was chosen based on their ability to compensate matrix effect. **Table 1** shows the MS/MS transitions selected, retention time (RT), mass spectrometric conditions and ILIS selected for each compound.

In the analysis of samples, the confirmation of positives was based on the accomplishment of *Q/q* ratios, with deviation lower than 30% between samples and reference standards, and RT error less than 0.1 min [26].

2.5. Method validation

The method performance was evaluated in terms of linearity, matrix effect, robustness, accuracy, precision and limits of detection and quantitation.

Calibration curves were freshly prepared prior to each analysis. *Linearity* was evaluated by analyzing standard solutions in solvent at eight concentration levels ranging from 0.1 to 50 μg L^{-1} (equivalent to 4–2000 ng L^{-1} in WW). Satisfactory linearity using weighted (1/*X*) least squares regression was considered when the correlation coefficient (R) was greater than 0.99.

Matrix effect was evaluated by analyzing matrix-matched standards at 25 μg L^{-1} and comparing with standards in solvent. *Robustness, precision* and *accuracy* analysis were evaluated by spiking WW at 100 and 1000 ng L^{-1}. Recovery was considered as acceptable when it was between 70 and 120%, with RSD within ± 20%.

Limits of quantification (LOQ) were calculated for a signal-to-noise (S/N) ratio of 10 using the quantitation transition in samples spiked at the lowest validation level. Additionally, at least one confirmation transition needed to show S/N ratio ≥ 3. *Instrumental limits of detection* (ILD) were set up for an S/N ratio = 3 in standards in solvent at the lowest concentration of the calibration line.

2.6. Collection of samples

Higher consumption of NPS and ID is often expected on weekends rather than weekdays when people unwind from weekday's routine. Therefore, for

monitoring the consumption of these type of substances through the analysis of WW, it was considered more suitable to collect WW samples on weekends [6].

24 h composite samples were taken from 8 different WWTPs by taking an aliquot of the influent stream every 15 min. Immediately, samples were frozen (−20 °C) and delivered to the laboratory. For each WWTP, a weekend-pooled sample was obtained by mixing Friday, Saturday and Sunday samples (1:1:1). Samples were analyzed by the fully validated UHPLC-MS/MS method.

Table 1. SRM acquisition mode in UHPLC-MS/MS. Retention time (RT), isotopically labelled internal standard (ILIS) used for correction, quantification (*Q*) and confirmation (*q1* and *q2*) transitions with cone voltage (CV) and collision energy (CE) associated.

Compound	RT (min)	ILIS	Precursor ion	CV (V)	*Q* transition		q_1 transition		q_2 transition		Q/q_1 (RSD %)	Q/q_2 (RSD %)
					Product ion	CE (eV)	Product ion	CE (eV)	Product ion	CE (eV)		
Amphetamine [a]	2.45	Amphetamine-d_6	136.1	10	119.0	10	91.1	20	65.0	40	0.8 (7)	44.5 (20)
Benzoylecgonine [b]	2.81	Benzoylecgonine-d_3	290.1	40	168.1	20	105.2	30	77.0	50	3.0 (4)	8.9 (5)
Butylone	2.73	Butylone-d_3	222.1	30	174.0	20	146.1	20	131.1	30	2.2 (9)	4.0 (7)
Cocaine [a]	3.13	Cocaine-d_3	304.2	40	182.2	20	82.0	30	77.0	50	2.9 (5)	15.5 (8)
Dimethylone	2.35	Methylone-d_3	222.1	40	147.0	20	72.1	20	91.0	30	0.8 (4)	2.7 (6)
Dipentylone	3.19	α-PVP-d_8	350.1	30	100.1	20	135.0	20	175.2	20	1.8 (4)	1.9 (7)
Ketamine	2.97	Ketamine-d_4	238.2	50	125.0	30	179.1	20	220.1	20	2.1 (12)	4.0 (7)
MDMA [a]	2.57	MDMA-d_5	194.1	10	163.0	10	135.0	20	105.1	20	2.7 (6)	3.3 (6)
MDPV	3.24	MDPV-d_8	276.1	35	126.0	30	175.1	20	149.0	30	2.0 (4)	1.3 (7)
Mephedrone	2.83	Mephedrone-d_3	178.1	40	145.0	20	160.1	10	91.1	30	0.6 (6)	237 (13)
Methamphetamine [a]	2.55	Methamphetamine-d_5	150.2	10	119.3	10	91.0	20	65.0	40	0.4 (5)	19.4 (10)
Methedrone	2.57	MDMA-d_5	194.1	40	161.3	20	176.1	10	146.1	30	0.9 (11)	3.4 (9)
Methoxetamine	3.11	Methoxetamine-d_3	248.2	50	121.1	30	175.0	20	203.0	10	1.0 (11)	0.7 (5)
Methylone	2.24	Methylone-d_3	208.1	30	160.0	20	132.0	30	91.1	40	2.8 (6)	17.6 (11)
N-ethcathinone	2.35	Methylone-d_3	178.1	20	117.1	10	130.0	30	160.1	10	0.6 (3)	0.1 (6)
PMMA	2.70	PMMA-d_3	180.1	40	149.0	10	121.1	20	91.1	30	1.2 (2)	9.5 (9)
α-PVP	3.19	α-PVP-d_8	232.2	10	105.1	30	91.0	20	126.0	20	0.2 (7)	0.6 (5)
3,4-DiMeO-α-PVP	3.18	Methoxetamine-d_3	292.1	30	221.1	20	151.1	30	126.0	30	1.0 (9)	1.8 (10)

Compound	RT (min)	ILIS	Precursor ion	CV (V)	*Q* transition		*q*1 transition		*q*2 transition		*Q*/*q*1 (RSD %)	*Q*/*q*2 (RSD %)
					Product ion	CE (eV)	Product ion	CE (eV)	Product ion	CE (eV)		
4-chloro-α-PPP	3.16	Methoxetamine-d_3	238.1	50	139.0	20	98.0	30	103.0	30	1.9 (5)	4.0 (7)
4-FMC	2.28	MDMA-d_5	182.0	30	149.0	20	103.1	30	164.0	10	7.4 (4)	0.8 (3)
4-MEC	2.98	Mephedrone-d_3	192.1	50	145.0	20	174.1	10	91.0	30	0.6 (2)	4.7 (4)
4-MePPP	3.03	Mephedrone-d_3	218.1	35	119.0	20	98.3	30	147.2	20	2.2 (2)	187 (12)
Amphetamine-d_6	2.45	-	142.2	10	93.0	20	-	-	-	-	-	-
Benzoylecgonine-d_3	2.81	-	293.3	40	171.1	20	-	-	-	-	-	-
Butylone-d_3	2.73	-	225.1	30	177.1	20	-	-	-	-	-	-
Cocaine-d_3	3.13	-	307.3	40	185.2	20	-	-	-	-	-	-
Ketamine-d_4	2.97	-	242.2	50	129.2	30	-	-	-	-	-	-
MDMA-d_5	2.57	-	199.1	10	165.1	10	-	-	-	-	-	-
MDPV-d_8	3.24	-	184.2	35	205.0	30	-	-	-	-	-	-
Mephedrone-d_3	2.83	-	181.1	40	145.1	20	-	-	-	-	-	-
Methamphetamine-d_5	2.55	-	155.2	10	92.1	20	-	-	-	-	-	-
Methoxetamine-d_3	3.11	-	251.2	50	124.1	30	-	-	-	-	-	-
Methylone-d_5	2.24	-	211.1	30	163.1	20	-	-	-	-	-	-
PMMA-d_3	2.70	-	183.1	40	121.1	20	-	-	-	-	-	-
α-PVP-d_8	3.19	-	240.2	10	91.0	20	-	-	-	-	-	-

[a] Illicit Drugs
[b] Benzoylecgonine is the main metabolite of cocaine.

3. Results and discussion

3.1. Exploring the capabilities of μLC-MS/MS

The potential application of μLC for the determination of ID and NPS in WW was tested and compared to conventional UHPLC in terms of chromatographic separation, injection volume, sensitivity and in-matrix reproducibility.

3.1.1. Chromatographic separation

Chromatographic separation was initially assessed with H_2O:MeOH mobile phases resulting in sparse peak shapes. Consequently, HCOOH was added to both mobile phases in order to enhance the formation of the protonated molecule and, therefore, to improve compound sensitivity. The best conditions were found adding 0.01% HCOOH to both H_2O and MeOH. Moreover, the addition of ammonium acetate at different concentrations was also considered. It was found that 5 mM of NH4Ac in aqueous mobile phase improved the peak shape for some compounds. However, it was thought to be causing over pressure in the μLC fluidics because of poor solubility in methanol (roughly 20 times less than in pure H_2O [27]). This behavior is not an issue of concern in conventional UHPLC systems because of the higher fluidics dimensions, but it becomes important when going down to micro-flow systems with capillary tubes. Finally, its utilization was avoided and only HCOOH was added to mobile phases.

The gradient selected for the appropriate elution of all compounds included in the study consisted on a first slow and steady increase in the percentage of organic phase (from 30% to 60% in 2 min) permitting good separation, especially for the cathinones and the amphetamine-like compounds. This was followed by an abrupt increase up to 90% of mobile phase B in 0.1 min. The latter favors the elution of less polar compounds of the matrix and results in a 5.50 min chromatographic run time.

For the μLC system, different flow rates, ranging from 0.5 to 3 μL min^{-1}, were tested to evaluate the sensitivity. The reduction of flow rate resulted in poor peak shape and lower sensitivity for highly polar and small compounds such as cathinones and amphetamines. In addition, run time increased significantly

because of dead-volumes playing an important role in micro-fluidic systems. No flow rates higher than 3 μL min^{-1} were tested since system pressure was close to the highest operating limit. Finally, 3 μL min^{-1} was selected.

The low flow rates applied in μLC result in increased sensitivity due to the higher ionization efficiency compared to conventional flow rates used in UHPLC. This was demonstrated by comparing the normalized peak areas (**Table 2**), where the increasing factor in sensitivity was between 6 and 53.1 times for the substances investigated. The hundred-fold reduction in flow rate is not only important for the sensitivity point of view. It also leads to a notable decrease in the consumption of organic solvents moving μLC systems towards a more green analytical chemistry.

Table 2. Comparison of UHPLC-MS/MS and μLC-MS/MS sensitivity (normalized response) and method performance (absolute area) for ID and NPS.

Compound	Normalized response (peak area per 10 pg of substance injected)			Absolute area		
	UHPLC (peak area/10 pg)	μLC (peak area/10 pg)	Sensitivity improvement factor (μLC/UHPLC)	UHPLC (peak area)	μLC (peak area)	Sensitivity improvement factor (μLC/UHPLC)
Amphetamine [a]	4111	218444	53.1	12333	218444	17.7
Benzoylecgonine [b]	289298	2002463	6.9	867894	2002463	2.3
Butylone	171688	1409698	8.2	515064	1409698	2.7
Cocaine [a]	432300	3363345	7.8	1296900	3363345	2.6
Dimethylone	117516	816537	6.9	352548	816537	2.3
Dipentylone	180165	1613366	9.0	540495	1613366	3.0
Ketamine	121797	1197573	9.8	365391	1197573	3.3
MDMA [a]	256935	2602112	10.1	770805	2602112	3.4
MDPV	171838	1031096	6.0	515514	1031096	2.0
Mephedrone	353980	3565801	10.1	1061940	3565801	3.4
Methamphetamine [a]	26482	866645	32.7	79446	866645	10.9
Methedrone	190383	3103025	16.3	571149	3103025	5.4
Methoxetamine	201885	1550649	7.7	605655	1550649	2.6
Methylone	95853	578751	6.0	287559	578751	2.0
N-ethcathinone	10101	354736	35.1	30303	354736	11.7
PMMA	165882	1815450	10.9	497646	1815450	3.6
α-PVP	54328	562469	10.4	162984	562469	3.5
3,4-DiMeO-α-PVP	124625	937913	7.5	373875	937913	2.5
4-chloro-α-PPP	107722	1103581	10.2	323166	1103581	3.4
4-FMC	117408	994430	8.5	352224	994430	2.8
4-MEC	44783	698796	15.6	134349	698796	5.2
4-MePPP	154231	1596327	10.4	462693	1596327	3.5

[a] Illicit Drugs
[b] Benzoylecgonine is the main metabolite of cocaine.

3.1.2. Injection volume and injection solvent composition

Injection volumes of 0.1, 0.2 and 0.5 µL in partial loop mode and 1 µL in full loop mode were tested in the µLC system. For appropriate response comparison, peak areas were normalized with the sample volume injected in the system. **Figure S2** shows the *peak area:injected volume* ratio for mephedrone, methoxetamine, α-PVP and dipentylone, as illustrative examples of the general trend observed for all compounds. The best *peak area:injected volume* ratio was found at an injection volume of 1 µL for all compounds.

Injection volume and sample extract composition are two strategic instrumental parameters to optimize during method development. Depending on the injection mode (full or partial loop), sample composition becomes of paramount importance for compound retention in the head of the column especially in micro-fluidic systems. When working in partial loop mode, the selected amount of sample is loaded and the rest of the loop is filled with weak washing solvent (H_2O) (see **Figure S3** in **Supporting Information**). Therefore, if the extract and weak solvent compositions are not similar, diffusion of matrix extract in the aqueous phase could occur causing poor retention of compounds, and spreading peaks throughout the chromatogram.

Sample extract composition also becomes an issue of concern because of the small column volume of the iKey device, which is nearly 0.35 µL and translates into a maximum suggested injection volume of 0.03 µL [28]. Working with an injection volume of 1 µL means overloading the chromatographic column with approximately 3,333%, which significantly compromises chromatographic resolution, especially for early eluting peaks. Thus the injection solution strength should be as low as possible (ideally, non-organic content). In this way, although the injected amount of sample is larger than the loading capacity, compounds are retained in the head of column and not eluted with injection solvent.

In conventional UHPLC systems, few microliters of sample are typically injected as well. However, the loading capacity of the chromatographic columns is significantly larger than µLC columns and, therefore, injection volume does not exceed the maximum recommended. Hence, higher organic compositions and

higher injection volumes could be used without compromising chromatographic separation.

3.1.3. Sensitivity comparison of µLC-MS/MS and UHPLC-MS/MS

A comprehensive comparison in terms of sensitivity was made between µLC-MS/MS and conventional UHPLC-MS/MS systems. For this purpose, a standard solution containing 10 µg L^{-1} of all compounds was injected in both instruments at the injection volumes previously selected, *i.e.* 1 µL and 3 µL, respectively. **Figure 1** shows the chromatograms corresponding to the *Q* transition of methoxetamine, α-PVP and PMMA in µLC-MS/MS and UHPLC-MS/MS as illustrative examples of the sensitivity differences.

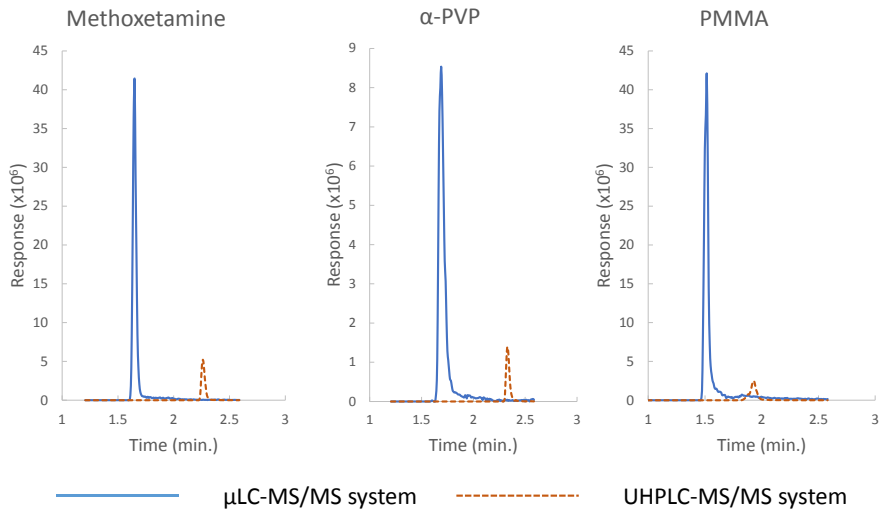

Figure 1. Chromatograms for methoxetamine, α-PVP and PMMA (10 µg L^{-1}) for both µLC-MS/MS (blue continuous-line) and conventional UHPLC-MS/MS systems (orange dashed-line).

Both, sensitivity enhancement provided by the technique and absolute method performance, have been evaluated. **Table 2**, shows a comparison of the peak area normalized per 10 pg of substance injected for both techniques as well as the ratio between normalized peak areas. It can be seen that the utilization of micro-flow techniques is more beneficial for the more polar compounds, such as

amphetamine, N-ethcathinone and methamphetamine, which showed an increase factor in sensitivity of 53.1, 35.1 and 32.7, respectively. For less polar compounds, such as methylone, MDPV, benzoylecgonine and dimethylone, sensitivity in μLC increased approximately 6 times. The average increasing factor in sensitivity from UHPLC to μLC systems was approximately 14.

The higher volume injected into a UHPLC system may be, however, an advantage. The overall method performance between μLC and UHPLC can be compared if the absolute peak areas are brought into comparison. Even though the optimized injection volume used in UHPLC was 3 times higher than in μLC (3 μL vs 1 μL), the overall sensitivity achieved with μLC methodology was still on average 4.5 times higher. Yet, it should be considered that the volume that can be injected in a UHPLC system is more flexible, and might be, in principle, increased to improve sensitivity. In order to reach comparable levels of overall method sensitivity with μLC, 14 μL of sample extract would be needed in UHPLC. However, an increase in injection volume may not always be beneficial because more matrix is also injected and can result in considerable issues related to matrix effects.

The increase in sensitivity associated to micro-flow techniques is highly valuable especially for compounds, such as NPS and some ID, that are present at very low concentration levels in water. Thus, μLC–MS/MS presents, in principle, strong potential for the monitoring of NPS consumption by means of WW analysis.

3.1.4. In-matrix reproducibility

μLC–MS/MS systems have been reported to be less matrix interfered than conventional UHPLC-MS/MS systems [29]. Due to its intrinsic low flow rate, the ionization efficiency is higher than in conventional ESI sources resulting in less ion suppression. However, this low matrix interference is referred solely to the ion suppression that can potentially occur in the ionization process. On the contrary, the effect of matrix was more severe on the chromatographic retention for the compounds included in this study.

Two randomly selected WW samples were spiked at 10 $\mu g\ L^{-1}$ to assess the robustness of the chromatographic separation as well as the effect on the matrix. **Figure 2** shows the chromatograms (*Q* transition) for the WW samples spiked

with PMMA, methoxetamine, ketamine, and methedrone. As shown, strong deviations in RT, up to roughly 0.4 min, were observed. The vast majority of the compounds showed similar behavior in the spiked samples. The variability in RT illustrates how matrix composition affects analyte retention in the chromatographic column. Again, injection volume plays a key role in μLC–MS/MS since a large overloading of the column could result in an altered retention capacity [28], which usually is not an issue of concern in UHPLC.

Since reproducibility of RT is of great relevance in all type of analysis, particularly in complex matrix samples, such as WW, where the confirmation of identity of the compounds may become problematic, the μLC–MS/MS methodology tested in this work was found not robust enough for the determination of NPS and ID in WW. Consequently, validation of the method and subsequent analysis of samples was only performed by UHPLC-MS/MS.

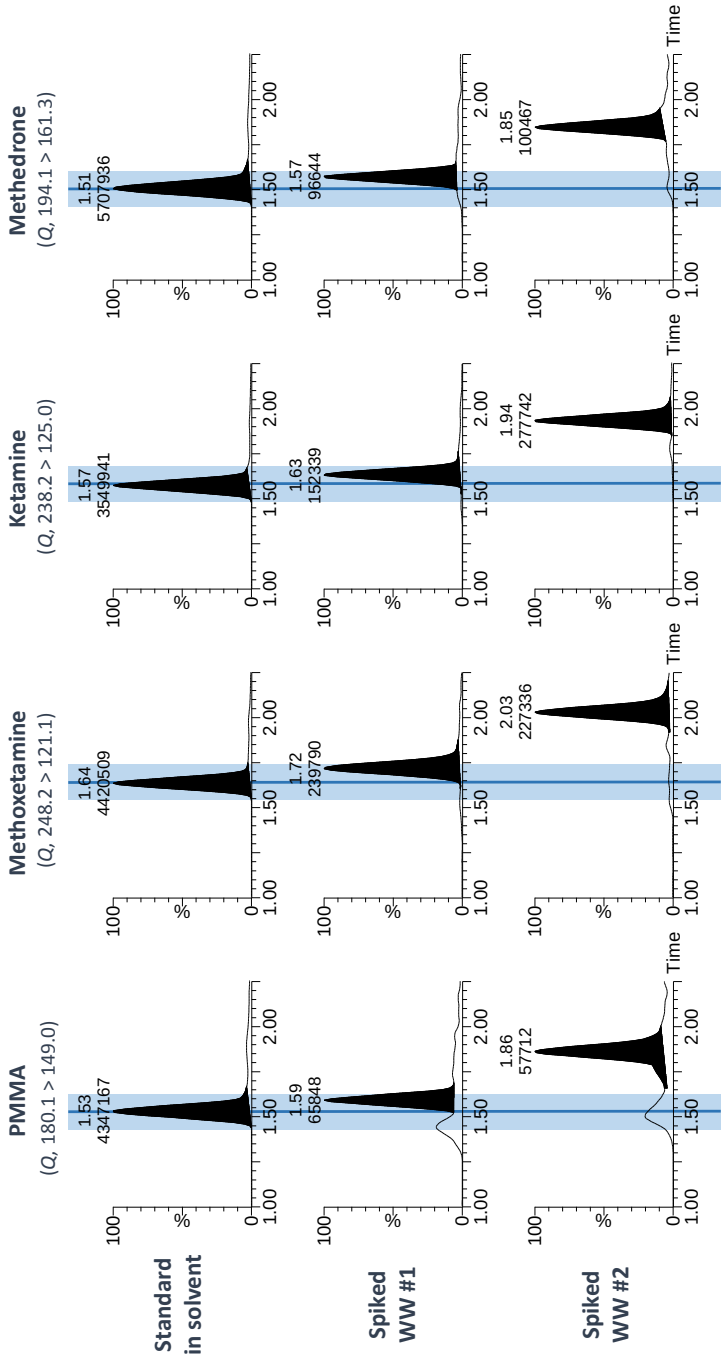

Figure 2. µLC-MS/MS chromatograms of PMMA, methoxetamine, ketamine and methedrone. *Q* transition monitored in Standard in Solvent (upper chromatogram) and 2 different wastewater samples (rest of chromatograms) as examples of the variation in RT. (Dark blue line indicates RT of the standard and the blue area shows the accepted deviation of RT in sample).

3.2. Method validation

The poor RT reproducibility of μLC–MS/MS, leads us to select UHPLC-MS/MS for subsequent method validation and real-world WW sample analysis. As several analytes were present in the "blank" samples used for validation, all the analytical data presented herein are blank-corrected.

Table 3 shows a summary of linearity, recovery, ILD and LOQ for the NPS and ID investigated. Good linearity within 0.1–50 μg L^{-1} with correlation coefficients (R) greater than 0.99 was observed in all cases. LOQs ranged from 3 (cocaine, benzoylecgonine, MDMA) to 70 (amphetamine) ng L^{-1} in raw WW, and were similar to those reported in the literature [5,6,30]. Precision and accuracy (n = 5) were evaluated by spiking 'blank' WW samples at two concentration levels (100 and 1000 ng L^{-1}). The results obtained for all compounds were satisfactory at both spiking levels, with recoveries between 75–114 % at the lowest level and 84–119 % at the highest level. Precision (RSD) was in the range 1 to 16% at the lowest spiked level and 2–11 % at the highest spiked level. It is noteworthy that recovery for benzoylecgonine was not calculated at the lowest level due to the high concentration of this chemical in the "blank" WW used for validation purposes. Matrix effects were evaluated using matrix-matched standards. The matrix extracted with SPE resulted in different rates of ion suppression, which were corrected by using appropriate ILIS.

Table 3. Instrumental limit of detection (ILD), limit of quantification (LOQ) and Correlation Coefficient (R) in linearity assay. UHPLC-MS/MS method validation results for ID and NPS in WW.

Compound	Recovery (RSD %)		ILD (fg)	LOQ (ng L^{-1} in WW)	Correlation coefficient (R)
	100 ng L^{-1}	1000 ng L^{-1}			
Amphetamine [a]	75 (16)	117 (6)	133	70	0.9936
Benzoylecgonine [b]	- [c]	90 (3)	13	3	0.9982
Butylone	96 (5)	95 (3)	132	6	0.9994
Cocaine [a]	120 (7)	114 (2)	16	3	0.9963
Dimethylone	100 (3)	97 (5)	129	20	0.9987
Dipentylone	105 (8)	107 (6)	68	6	0.9986
Ketamine	102 (8)	92 (3)	80	4	0.9990
MDMA [a]	93 (1)	100 (2)	32	3	0.9994
MDPV	88 (17)	96 (7)	88	21	0.9990
Mephedrone	109 (5)	112 (3)	21	5	0.9909
Methamphetamine [a]	114 (14)	119 (4)	51	21	0.9988
Methedrone	92 (6)	94 (5)	42	6	0.9977
Methoxetamine	107 (9)	105 (5)	53	5	0.9996
Methylone	104 (3)	103 (5)	57	4	0.9967
N-ethcathinone	76 (14)	88 (7)	42	41	0.9978
PMMA	111 (6)	99 (5)	19	10	0.9927
α-PVP	101 (15)	95 (11)	103	43	0.9988
3,4-DiMeO-α-PVP	103 (4)	99 (7)	142	9	0.9954
4-chloro-α-PPP	85 (16)	84 (8)	36	17	0.9957
4-FMC	86 (11)	92 (9)	31	30	0.9971
4-MEC	88 (5)	96 (5)	49	5	0.9956
4-MePPP	85 (5)	93 (5)	110	18	0.9970

[a] Illicit Drugs

[b] Benzoylecgonine is the main metabolite of cocaine.

[c] Recovery of Benzoylecgonine in lowest spiked level not calculated because of the high concentration of *Blank* sample

3.3. Sample analysis

Pooled weekend WW samples were analyzed by means of the validated UHPLC-MS/MS methodology described above.

All the monitored ID were confirmed and quantified in any of the samples analyzed, with cocaine (and its main metabolite benzoylecgonine) and MDMA being found in all the analyzed samples (**Table 4**). Amphetamine was confirmed and quantified in all samples except one where it was found below the LOQ level. Methamphetamine showed a great variability in concentration levels, being below LOQ in half of the samples. Regarding NPS, ketamine and dipentylone were confirmed and quantified in some WW. It is noteworthy to highlight the presence of ketamine in all samples above LOQ level, suggesting an established consumption in the assessed communities. The same trend for ketamine was observed in several Italian cities from 2008 to 2014. Despite ketamine being an emerging NPS at that moment, an increase on its consumption was detected in all cities included in the study [31]. The EMCDDA defines NPS as 'newly misused' substances; therefore, the persevering consumption of ketamine makes this substance not strictly fitting this criterion. However, in this study ketamine has still been considered as NPS because of its novelty in comparison with the traditional ID (cocaine, amphetamine, etc.). Butylone, mephedrone, methedrone and methylone were also found in three samples.

Confirmation of compound identity required the accomplishment of RT (deviation ≤ 0.1 min) and Q/q ratios (deviation ≤ 30% in at least one out of the two confirmatory transitions) in comparison with the reference standard. **Figure 3a** shows an example of MDMA confirmation (full agreement with reference standard). The problematic identification of mephedrone in one of the samples is also illustrated in this figure (Figu**re 3b**). The RT in sample agreed with the quality control (spiked "blank" sample), but only one confirmatory transition was observed (Q/q_1), because the third transition (Q/q_2) available had very unfavorable ion ratio (see **Table 1**) and could not be detected. Unfortunately, the deviation of Q/q_1 ratio was above 30%, and mephedrone could, therefore, not be confirmed. Additional analysis (*e.g.* modifying

chromatographic conditions, additional clean-up, etc.) would be required for confirmation of that potential positive.

Table 4. Concentration (in ng L^{-1}) of five ID and metabolites and six NPS found in influent WW from eight WWTPs.

Compound		Location WWTP							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
		Concentration in raw wastewater [a] (ng L^{-1})							
ID	Amphetamine	716	7565	194	307	1527	382	1564	<LOQ
	Benzoylecgonine [b]	4594	10558	3416	2724	5783	5268	2232	297
	Cocaine	1165	2316	585	809	1177	961	206	31
	MDMA	250	110	28	73	316	150	144	216
	Methamphetamine	159	<LOQ	34	<LOQ	296	<LOQ	<LOQ	406
NPS	Butylone	-	-	-	-	-	-	d	-
	Dipentylone	-	-	6.4	-	-	-	-	-
	Ketamine	38	33	8.0	57	79	23	22	9.6
	Mephedrone	d	-	-	-	-	-	-	-
	Methedrone	-	d	-	-	-	-	-	-
	Methylone	-	-	-	-	-	-	d	-

[a] <LOQ: Below limit of quantification

[b] Main metabolite of cocaine

d: Compound detected but not confirmed because of deviations in *Q/q* ratios

-: Not Detected

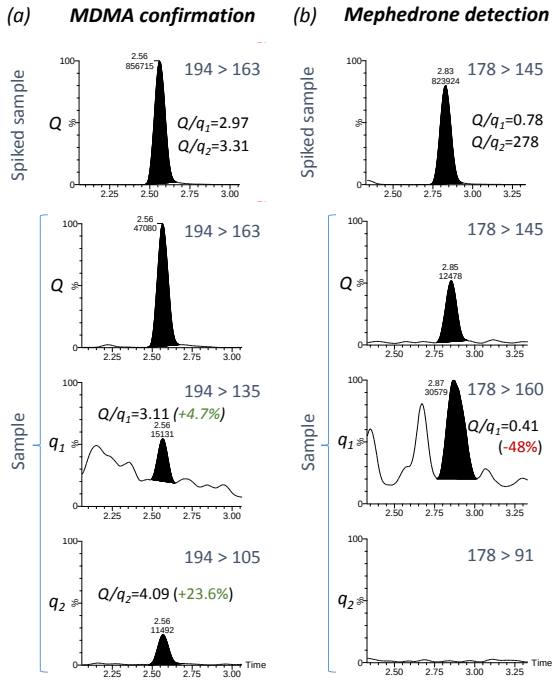

Figure 3. Selected examples of UHPLC-MS/MS analysis of wastewater samples. (a) MDMA confirmation in wastewater. (b) Mephedrone detection in wastewater. The *Q* chromatogram of the spiked sample is shown, together with the *Q/q* ratios (top). The *Q, q1* and *q2* chromatograms of the real-world sample and the *Q/q* ratios in the sample are also shown (bottom). Note that MDMA was confirmed by compliance of the *Q/q* ratios (maximum tolerance ± 30%), but mephedrone could not be confirmed (*Q/q* deviation −48%).

Despite some NPS have been identified/quantified (butylone, dipentylone, ketamine, mephedrone, methedrone and methylone), the most abundant drug found in WW was cocaine (and its main metabolite benzoylecgonine) followed by amphetamine, suggesting the consumers preference for known ID rather than the NPS investigated. Among the NPS detected, some compounds have been also found by other studies. Thus, Bade *et al.* reported the presence of mephedrone, methylone and MDPV (not detected in this study) in WW from different European cities in 2017 [5]. Senta *et al.* found methedrone, methylone, mephedrone and ketamine in Croatia [32]. In addition, butylone, mephedrone, methylone, α-PVP and MDPV (among other compounds not included in this

study) were also reported by Fontanals *et al.* in the east coast of Spain [33]. The fact that MDPV and α-PVP were not found in the wastewaters analyzed in this work might be associated to the ever changing NPS market, which is constantly evolving to look for new substances.

It is worth to notice that dipentylone has been found in WW for the first time. Further studies in the forthcoming years will be required to assess whether dipentylone has appeared in the NPS market to become a largely consumed substance or its presence in WW is due to an acute peak of consumption. The results obtained in this work reveal that WW analysis has potential for the assessment of NPS consumption in a community, as up to 6 NPS have been found, one of them for the first time.

3.4. Strengths and limitations of micro-LC for wastewater-based epidemiology applications

Recently developed integrated μLC-ESI chip interfaces have revolutionized micro-LC technique. The older capillary chromatography instruments were highly user-dependent in terms of assembling pieces; however, the new integrated devices provide similar instrumental robustness as conventional UHPLC systems. This strongly facilitates their utilization, enabling analytical applications with enhanced sensitivity [16].

Moving from UHPLC to micro-LC separation require some strategic method rearrangements since sample composition and injection volume play an important role in micro-LC. Furthermore, the quality of solvents and mobile phase additives solubility are crucial in micro-LC separations. For the determination of NPS and ID in WW, small arrangements such as working with low percentage of organic solvent in the sample extract and in full-loop injection mode led to a notable increase in sensitivity using only a few millilitres of WW sample for analysis.

Wastewater is a highly complex matrix for the analysis of low concentrated substances such as ID and NPS. Besides, WW composition can vary considerably between locations, or even between days within the same location. In this work, the tested μLC–MS methodology was greatly affected by the sample matrix

resulting in bad RT reproducibility, which however did not affect to conventional UHPLC-MS/MS. A major issue is considerable sensitivity improvement observed for μLC–MS/MS in comparison to conventional UHPLC–MS/MS with up to 53-increase fold (average increase for all compounds, 14), especially for early-eluting compounds, such as cathinones and amphetamines. The overall method performance using μLC–MS/MS also improved, up to 17.7- increase fold (4.5 times on average).

The observed increase in sensitivity makes micro-fluidic techniques an attractive approach for WBE applications in the near future. With low prevalence of consumption, high variability of choice for consumers and low doses for most NPS, an enhanced sensitivity might be the difference between detection or not detection in WW. Further investigation to get better reproducibility across samples (*e.g.* modifications in sample treatment) will allow to monitor controlled and non-controlled substances reaching lower detection limits than those provided by UHPLC–MS methodologies. Manufacturers progresses, such as the development of column chemistries more inert to the matrix with higher injection volume capacities, would facilitate the application of this technique as well. The high cost of the iKey columns and of the high-quality solvents required in μLC are also relevant issues that need to be considered. A major point is that μLC techniques represent a step forward into a greener analytical chemistry thanks to the less amount of sample and organic solvents needed.

4. Conclusions

A simultaneous sensitive analytical methodology, based on SPE and UHPLC-MS/MS, has been developed for quantitative determination of 22 compounds, including ID, NPS and some of their main metabolites, in wastewater samples. The sample throughput has been improved in comparison with other reported methodologies by reducing the amount of sample loaded in the cartridge. The small sample volume required for analysis (5 mL) notably facilitates sample collection and shipping in large sampling campaigns. The methodology has been successfully applied to pooled weekend WW samples in which cocaine, amphetamine, and MDMA were the most abundant drugs. Six NPS (ketamine, dipentylone, butylone, mephedrone, methedrone and methylone) were also found, with ketamine being present in all analyzed samples.

Moreover, the potential application of μLC-MS/MS in WBE studies has been evaluated and thoroughly compared to UHPLC-MS/MS. The adaptation of UHPLC-MS/MS methodologies to μLC–MS/MS require to carefully consider sample matrix composition and to optimize injection volume since these parameters playing a key role in the chromatographic separation. The matrix effect on compound chromatographic retention was found an issue of concern because of the lack of RT reproducibility. However, the substantial increase in sensitivity, the high throughput of samples because of the low analysis time and the steps forward to a greener analytical chemistry make μLC-MS/MS a promising tool for future applications in wastewater-based epidemiology.

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Supporting Information

In this section, information about the chemicals and materials used as well as a discussion regarding the evaluation and optimization of the extraction procedure can be found. Additionally, figure S1 presents a summary of the sample treatment, figure S2 includes supporting data for the evaluation of injection volume and figure S3 depicts the sample-solvent distribution in the injection valve.

Supplementary data to this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2019.05.051 and in this chapter after the section “References”.

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Supporting Information

S1. Chemicals and Materials

In total, 22 ID, NPS and main metabolites were included in this study. Amphetamine, benzoylecgonine (BE), butylone, cocaine, ketamine, mephedrone, methamphetamine, methedrone, methoxetamine, methylenedioxypyrovalerone (MDPV), methylone, N-ethylcathinone, 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxy-N,N-dimethylcathinone (bk-MDDMA), 4-methyl-α-pyrrolydinopropiophenone (4-MePPP) and α-pyrrolidinopentiophenone (α-PVP) were purchased from Cerilliant (Round Rock, TX, USA). Dimethylpentylone (bk-DMBDP), ρ-methoxymethamphetamine (PMMA), 3,4-dimethoxy-α-pyrrolidinopentiophenone (3,4-DiMeO-α-PVP), 4-chloro-α-pyrrolydinopropiophenone (4-C-α-PPP), 4-fluoromethcathinone (4-FMC) and 4-methylethcathinone (4-MEC) were purchased at Cayman Chemical Co. (An Arbor, MI, USA). Isotopically-labelled reference standards were used as surrogate internal standards for quantitation. Amphetamine-d_6, BE-d_3, butylone-d_3, cocaine-d_3, ketamine-d_4, mephedrone-d_3, methamphetamine-d_5, methoxetamine-d_3, MDPV-d_8, methylone-d_3, MDMA-d_5, and α-PVP-d_8 were purchased from Cerilliant (Round Rock, TX, USA). PMMA-d_3 was purchased at Cayman Chemical Co. (An Arbor, MI, USA).

Methanol (MeOH) LC-MS grade, acetonitrile (ACN) LC-MS grade , ammonium acetate (NH_4Ac), formic acid (HCOOH) (LC-MS additive grade, >99%) were acquired from Scharlau (Barcelona, Spain). HPLC-grade water (H_2O) was obtained by purifying demineralized water in a Milli-Q plus system from Millipore (Bedford, MA, USA).

Standard stock solutions of each compound were prepared at 100 – 1000 mg L^{-1} in MeOH or ACN. Intermediate solutions were prepared by diluting the stock solution with MeOH. Infusion solutions of individual standards were prepared at a concentration of 100 μg L^{-1} in MeOH:H_2O (50:50, *v/v*). Mixed solutions containing all analytes were prepared by appropriate dilution of intermediate solutions with Milli-Q water, and were used for preparation of the calibration standards, spiking samples in the validation study and as internal quality

controls. Individual stock solutions of isotope-labelled standards were prepared in MeOH or ACN at a concentration of 10 - 200 mg L^{-1}. A mixed isotope-labelled standard working solution at 50 µg L^{-1} was prepared in MeOH:H_2O (20:80, *v/v*) and used as surrogate internal standard. All standard solutions were stored in amber glass bottles at -20ºC.

Solid Phase Extraction (SPE) cartridges tested were Oasis HLB 3 cm^3 (60 mg) and Oasis MCX 3 cm^3 (60 mg) from Waters (Milford, MA, USA). Nylon filters of different sizes (25, 17, 13 and 4 mm) and pore dimensions (0.45 and 0.22 µm) tested were supplied by Teknokroma (Barcelona, Spain), Membrane solutions (Plano, TX, USA) and Supelco (Munich, Germany).

S2. Sample treatment and matrix effects. Evaluation and Optimization of extraction procedure

The analytical methodology developed was based on previous studies on the analysis of ID and NPS in WW by UHPLC-MS/MS (1-3). In the present work, both ID and NPS were included in one unique method, and additional NPS were included in the target compounds list in order to widen the monitoring capabilities in the field of WBE. Furthermore, the sample volume required for analysis was reduced. Several processes included in sample treatment were evaluated: solid phase extraction, evaporation and solids removal.

The presence of solid particles in WW is of concern when performing SPE. Also, the potential particulate that could occur in the reconstituted extract after SPE and evaporation should be eliminated when working in UHPLC-MS/MS or µLC-MS/MS instruments. Therefore, the potential losses of analytes in the filtration step were evaluated pre- and post- SPE. Milli-Q H_2O was spiked at two concentrations, one representing raw WW (10 ml of standard mixture in H_2O at 2.5 µg L^{-1}) and the other representing WW extract (200 µL of standard mixture in H_2O at 20 µg L^{-1}). Filtration using Nylon filters of different sizes (25, 17, 13 and 4 mm) and pore dimensions (0.45 and 0.22 µm) was evaluated. It was observed that filtering 'raw WW' produced certain losses for some analytes, and therefore, it should be avoided. In order to avoid potential clogging of SPE cartridge, it was decided to centrifuge raw WW at 6000 rpm for 5 min. In the case of 'WW extract',

the recoveries observed for all analytes were satisfactory when using Nylon filters of 4 mm with 0.22 μm of pore size.

The evaporation of the eluting solvent was assessed. The utilization of a vacuum evaporator (miVac DUO Concentrator, Genevac) and the evaporation of solvent under N_2 flow both at 40ºC were compared. It was observed that the utilization of vacuum evaporation resulted in better and more reproducible recoveries than under a N_2 steam.

SPE was optimized in terms of cartridge chemistry (HLB or MCX), elution volume, matrix interference and cleaning. The first step optimized was the amount of MeOH required for the elution of all compounds of interest retained in the cartridge. Elution of loaded standard mixture was performed in consecutive aliquots of 500 μL up to a volume of 5 mL. It was observed that the vast majority of compounds were quantitatively eluted within the first fraction in both HLB and MCX cartridges, and therefore elution with 1 mL MeOH was selected to assure the quantitative extraction of analytes from the cartridge. In comparison with previous methodologies where SPE cartridges were normally eluted with 5 mL of MeOH, the proposed methodology reduces the amount of organic solvent required for cartridge extraction, which can be seen as a contribution towards green chemistry.

Additionally, both HLB and MCX cartridges were tested for extraction of spiked WW applying different cleaning steps. The best performance was observed for HLB cartridges and a subsequent washing step with 50 mL of Milli-Q water. The effect of washing step was evaluated and no analyte losses were observed. Also, the effect of loading 5, 20, 50 and 100 mL of WW was assessed. The last two cases seemed to over-load the cartridge resulting in poor recoveries. Additionally, the amount of matrix loaded with 20 mL of sample produced a substantial ion suppression for the majority of compounds studied. The best results were obtained loading 5 mL of WW sample, followed by 50 mL pure H_2O cleaning of the cartridge. In wide sampling campaigns, the delivery of samples from WWTPs to the analytical laboratory is one of the issues of concerns because of the inconveniences of using large-volume samples, and the high costs of shipping the

samples. The methodology developed in this work requires less volume of samples than previously reported (1-3). In addition, the sample throughput of the methodology is higher since less time is required for sample loading.

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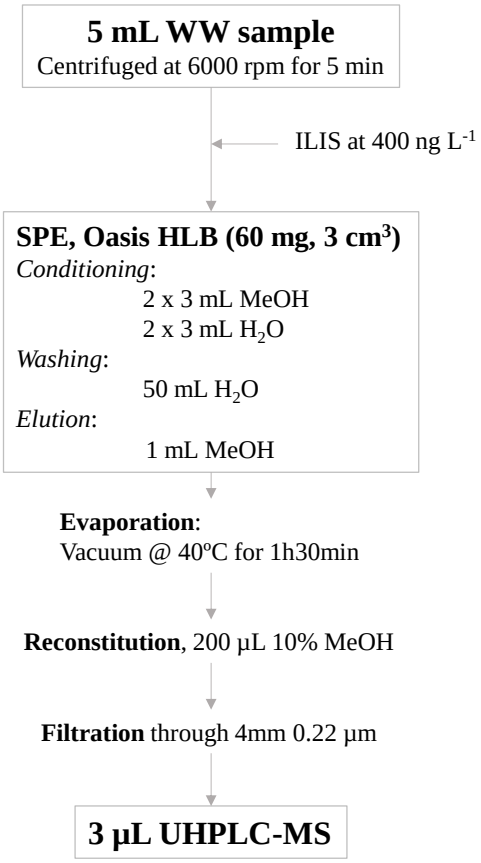

Figure S1. Flow chart of the experimental procedure for determination of NPS and ID in WW.

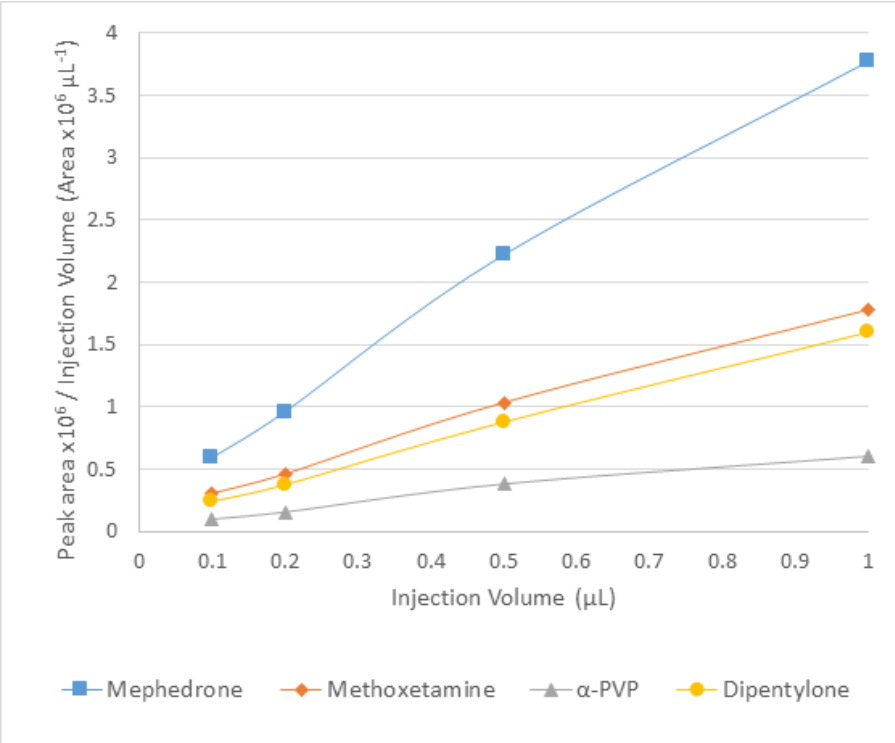

Figure S2. Comparison of compound response per microliter injected (*y*-axis) depending on injection volume (*x*-axis) in the µLC-MS/MS system.

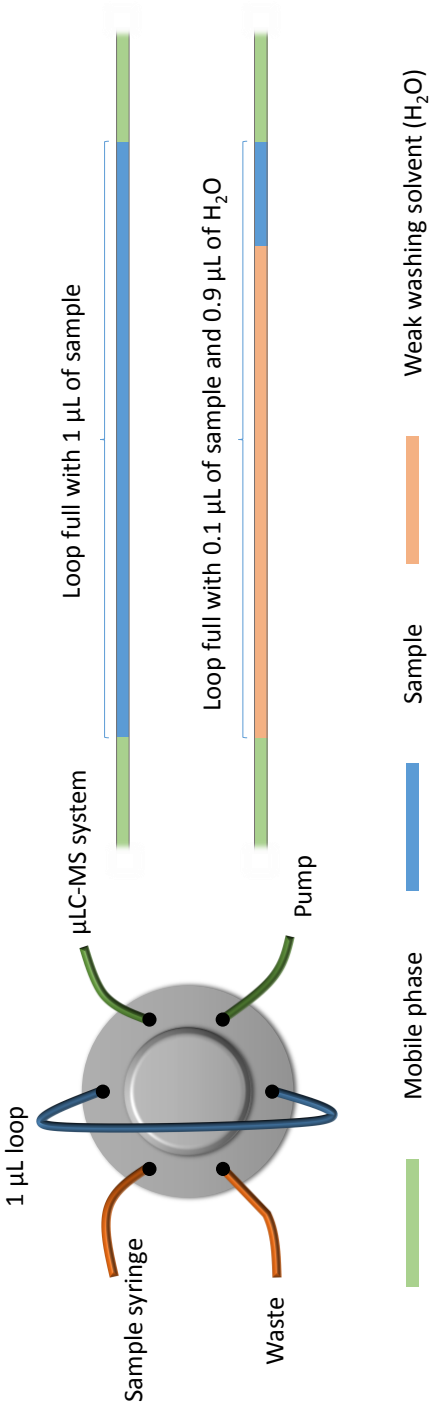

Figure S3. Comparison of full loop and partial loop loading of sample and solvents

Chapter 5.2.3. Scientific Article 10

PERSPECTIVES AND CHALLENGES ASSOCIATED WITH THE DETERMINATION OF NEW PSYCHOACTIVE SUBSTANCES IN URINE AND WASTEWATER – A TUTORIAL

Lubertus Bijlsma, Richard Bade, Frederic Been, Alberto Celma and Sara Castiglioni

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Tutorial

Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial

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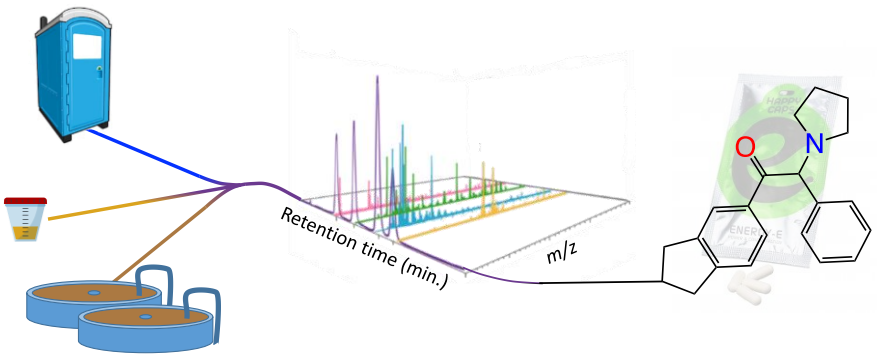
Graphical Abstract



Highlights

- Determination of NPS in urine and wastewater provide timely complementary information.
- Sensitive targeted methodologies play an important role in monitoring NPS use.
- HRMS-based analytical strategies permit widening the scope of NPS monitored.
- Identification capabilities can be improved by the MS acquisition workflow applied.
- Ion mobility separation is an innovative technique that smooths identification.

Keywords

New psychoactive substances; Biological samples; Wastewater-based epidemiology; Monitoring strategies; Mass spectrometry; Ion mobility separation.

Abstract

New psychoactive substances (NPS), often designed as (legal) substitutes to conventional illicit drugs, are constantly emerging in the drug market and being commercialized in different ways and forms. Their use continues to cause public health problems and is therefore of major concern in many countries. Monitoring NPS use, however, is arduous and different sources of information are required to get more insight of the prevalence and diffusion of NPS use. The determination of NPS in pooled urine and wastewater has shown great potential, adding a different and complementary light on this issue. However, it also presents analytical challenges and limitations that must be taken into account such as the complexity of the matrices, the high sensitivity and selectivity required in the analytical methods as a consequence of the low analyte concentrations as well as the rapid transience of NPS on the drug market creating a scenario with constantly moving analytical targets. Analytical investigation of NPS in pooled urine and wastewater is based on liquid chromatography hyphenated to mass spectrometry and can follow different strategies: target, suspect and non-target analysis. This work aims to discuss the advantages and disadvantages of the different data acquisition workflows and data exploration approaches in mass spectrometry, but also pays attention to new developments such as ion mobility and the use of *in-silico* prediction tools to improve the identification capabilities in high-complex samples. This tutorial gives an insight into this emerging topic of current concern, and describes the experience gathered within different collaborations and projects supported by key research articles and illustrative practical examples.

1. Introduction

New psychoactive substances (NPS) are continually evolving and introduced in different ways in the drug market. The NPS retail market is characterized by its dynamic nature and the large number of substances covering a broad range of drug categories [1,2]. Whereas most NPS disappear after a short time, others seem to establish a niche market [2,3]. They are often introduced as legal substitutes for known controlled drugs, but also explored for their novel effect. Some substances have been known for years and are now misused for recreational purposes, but most NPS are newly synthesized with little or no safety data regarding their short or long-term toxicity. Furthermore, purity and composition of products containing NPS are often not known, which places users at an even higher risk compared to well-known conventional illicit drugs [1,2]. The NPS market is extremely diverse and differs between countries. Governments have responded in different ways to the NPS market, but have not been able to act upon all the NPS which have emerged in an effective way in terms of penalizing its supply and use [4]. Hence, NPS continue to cause public health problems [5,6] and challenge healthcare professionals, toxicologists and policymakers in terms of identification, prevention, treatment and control.

The Early Warning Systems (EWS) established by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), Europol and the United Nations Office of Drugs and Crime (UNODC) play a key role in collecting data on new NPS appearing on the market. This information together with indications of the health and social risks associated with these substances is pivotal to respond to the emergence of NPS [7]. Analytical chemistry has a prominent role in gathering more thorough data which allows to better understand the situation of NPS use in the population. To complement the existing sources of information and improve our knowledge about the categories and characteristics of NPS present on the market, the application of appropriate analytical strategies is of utmost importance.

The discovery and characterization of new substances in commercially available products and drug seizures is an important source of information for EWS. Since

reference standards for unambiguous confirmation of the identity are often not available, a combination of several techniques, such as nuclear magnetic resonance (NMR), liquid chromatography (LC) coupled to high resolution mass spectrometry (HRMS), gas chromatography mass spectrometry (GC-MS) and X-ray crystallography, is normally applied [8–11]. Although there is a correlation, the identification of new substances in seized products mainly gives information on the NPS available on the market rather than information on the prevalence of use. Therefore, the analysis of biological samples is needed, but this implies a different analytical strategy to deal with the complexity of the matrix and the low analyte concentrations normally present in the samples [12–14].

The analysis of biological samples can be considered a frontline in the detection of consumed NPS. Samples of individuals can be collected from, for example, hospital emergency rooms, drug testing campaigns or post-mortem examinations, where concentrations of some NPS in acute intoxications may be relatively high. This may facilitate the identification of hitherto unknown intoxicants by means of the abovementioned analytical techniques [3]. However, it does not give a full picture of NPS use within a community, rather individuals, and the analyses of many samples required to have a wider picture is time consuming and expensive. In contrast, pooled urine and urban wastewater can anonymously provide information of many people in one single aggregated sample. Although the dilution factor can be rather high in these matrices, for example dilution of the sample with urine of non-consumers or water used in households and industry, it has demonstrated its utility for community-wide monitoring of illicit drug use and showed possibilities for getting complementary insight into the consumption and diffusion of NPS use [15–20].

Liquid chromatography hyphenated to tandem mass spectrometry instruments (LC-MS/MS) with triple quadrupole mass analyzers (QqQ) or hybrid HRMS/MS systems are the preferred analytical techniques that have the required high sensitivity and selectivity to deal with the challenges related to the screening of NPS in pooled urine and wastewater. Furthermore, the polar characteristics of most NPS and their metabolites, as well as the sample matrix, make them compatible with these techniques. This article aims to discuss the advantages

and disadvantages of relevant mass spectrometry (MS) data acquisition workflows and data exploration approaches to confront the low analyte concentrations and ever-changing NPS market and will be supported using key research articles and illustrative practical examples. This tutorial is not intended to be an extensive review of the existing literature, but to give an insight into this timely topic and describes the experience gathered within different collaborations and projects. It also pays attention to new developments such as ion mobility separation (IMS) and the use of *in-silico* prediction tools to improve the identification capabilities.

2. Sample collection and sample treatment

Well-designed protocols for sample collection and storage, and versatile sample treatment of pooled urine and wastewater are essential for getting data that provide meaningful information on NPS use. The collection of anonymous pooled urine samples from portable street urinals has recently demonstrated its utility to detect the use of recreational drugs, including NPS [18,19]. Generally, multiple samples are taken from various urine reservoirs, over a 12-h period, and then mixed to form pooled urine samples. This sampling method can be applied in cities where stand-alone urinals are routinely used at weekends [19], but can also be used for monitoring specific night time settings or recreational events such as music festivals [20–24]. Sampling urine aliquots from urinals ensures the collection of anonymous and representative samples and results may reflect the direct use of NPS. Yet, some limitations are related to the fact that urinals are designed for male use only and normally have no 'flushing' mechanism [25]. Thus, the number of contributors to the samples is unknown and, although quantitative analysis is possible, the comparison of concentrations gives little additional insight rather than a qualitative overview of the actual use of a certain drug compared to the other substances quantified in that specific sample.

Wastewater analysis may circumvent these limitations by providing anonymous population-normalized information of an entire community and has recently been explored to gather information on NPS use [3,15,17,26,27]. The successful application of wastewater-based epidemiology for assessing spatial differences and temporal changes in illicit drug use has been demonstrated [28,29] where population-normalized data can be calculated taking into account the measured concentration, the daily flow rate of sewage and the number of people connected to the wastewater treatment plant (WWTP) [28,30]. Specific sampling protocols have been developed to obtain representative 24-h composite wastewater samples collected at the inlet of a WWTP [28]. In addition, a standardized questionnaire facilitates the collection of relevant meta-data such as the daily flow rate of sewage and the number of people connected to the WWTP [31]. This meta-data allows quantitative population-normalized information for a limited number of target NPS to be explored. The information provided by wastewater

analysis can be integrated with existing epidemiological data because of the unique ability to provide objective, updated and nearly real-time information on drug use [16,32].

One sampling technique not yet fully explored but with potential for monitoring NPS in wastewater is passive sampling [33], which ensures the concentration of analytes from longer periods (days or weeks) and increases the possibility to detect substances with low prevalence of use. The main advantage is that passive samplers, consisting of polymeric-based sorbent material, deployed for longer periods, can accumulate trace analytes on the sorbent during this period. Moreover, as some NPS might be consumed sporadically (and thus might not always be present in wastewater), one does not need to collect multiple wastewater samples, which all eventually need to be processed *i.e.* increasing labor costs. Hence, this technology offers practical and economic advantages for gathering long-term data. But it has also some challenges related to calibration and quantification, since they require knowledge about uptake and diffusion of the different substances and are subject to the variability associated with NPS stability and environmental factors (*e.g.*, flow rates, biofouling) [33,34]. The uptake of target analytes on sorbent materials needs, therefore, to be determined prior to deployment in the sampling site.

Stability of NPS is an important aspect of sample collection for both pooled urine and wastewater analysis. While specific stability studies in pooled urine samples are lacking, they have been carried out on urine samples for forensic toxicology purposes. Metabolites of synthetic cannabinoids have been shown to be stable up to 14 days when refrigerated [35]. Many synthetic cathinones, benzodiazepines and amphetamine-type derivatives are very stable under freezing (−20 °C) storage conditions for months-years. However, when stored at room temperature or even refrigerated, degradation of these compounds can occur within days [36–38]. Therefore, it is recommended to freeze pooled urine samples immediately upon collection to avoid degradation. Regarding wastewater, it has been shown that acidification to pH 2 improves the stability in both filtered and unfiltered wastewater for up to 14 days for a wide variety of NPS such as cathinones, phenethylamines, opioid-derivatives and amphetamine-

like stimulants [39]. If samples cannot be acidified, it is recommended that they are kept either refrigerated (4 °C) or frozen (−20 °C) for no longer than one week prior to sample processing [39–42]. Several synthetic cannabinoids have been shown to be unstable at pH 2 and in raw wastewater *i.e.* the hydroxypentyl metabolites of JWH-122, AM-2201, RCS-4 and JWH-073, while JWH-018 N-pentanoic acid, JWH-073 N-butanoic acid and JWH-018 N-5-hydroxypentyl were stable at room temperature for up to 24 h [42]. Moreover, the use of sodium metabisulfite as a preservative has been recommended to improve the stability of synthetic cannabinoids [43].

A non-selective and versatile sample preparation protocol for the enrichment and clean-up of samples capable of retaining a wide range of NPS with broad physicochemical properties is preferred and applied by the vast majority of reported studies. Pooled urine samples are usually treated by performing a hydrolysis step to cleave drug-glucuronide conjugates with β-glucuronidase and arylsulfatase prior to solid-phase extraction (SPE), liquid-liquid extraction (LLE) and/or dilute and shoot techniques [21,44,45], while wastewater samples do generally not require this hydrolysis step due to in-sewer deconjugation [46–50] and are normally filtered and solid-phase extracted [17], although a less labor-intensive and quicker preparation procedure following the QuECHeRS principle has also been applied [51]. In order to cover the broadest range of substances possible, multiple SPE cartridges or cartridges consisting of several layers with different stationary phase chemistries can be used [27,52]. The use of more cartridges implies several separate extractions, yet these can be optimized to specific NPS categories of interest such as cathinones or synthetic cannabinoids [15,21]. Typically, cartridges containing polymeric-based SPE sorbents with reversed phase (RP) properties built of generic hydrophilic and lipophilic balanced monomers or strong cation-exchange mixed mode sorbents incorporating RP copolymers are used. For the latter, samples should be acidified to pH 2–3 to ensure that the analytes are positively charged during extraction [53]. This especially aids the recovery of cathinones, amphetamine-like stimulants, opioid derivatives and phenethylamines [21,39,45,54,55]. Online SPE has also been utilized for a limited number of NPS using a RP cartridge, with

satisfactory recovery (*i.e.* 70-120%) [56]. LLE has been shown to aid in the detection of synthetic cannabinoids in pooled urine [57] and wastewater [43,58]. For wastewater studies, it is important to note that the removal of the solid fraction through filtration can greatly affect the overall recovery of synthetic cannabinoids due to their lipophilicity. Therefore, when performing wastewater analysis, both the aqueous and particulate fraction should be extracted together for optimal recovery of cannabinoids.

Although both pooled urine and wastewater analyses incorporate SPE, there is a much lower pre-concentration factor needed for pooled urine, with initial volumes of 1–2 mL, due to the generally higher concentrations found [23,45,57]. Furthermore, lower pre-concentration results in less matrix effects and potentially an improved chromatographic performance. Higher pre-concentration factors in wastewater are commonly applied to deal with the very low concentrations of NPS expected in these samples. However, this can also result in strong matrix effects due to the pre-concentration of unremoved components present in the sample extract. Matrix effects are alterations of the MS signal (enhancement or suppression), which have been linked to co-eluting interferences such as proteins, lipids, sugars or salts, that affect the ionization process [59]. Frequently, isotopically labelled internal standards (ILIS) are used as surrogates and added to samples prior to processing (*i.e.*, SPE) or analysis (in the case of dilute and shoot approaches applied in pooled urine analysis), to account for potential matrix effects, but also to correct for potential errors due to sample preparation. Ideally, ILIS of the corresponding NPS are used as they are supposed to be affected in a similar manner as their non-labelled counterparts. However, ILIS are often expensive and not always commercially available, especially in the case of NPS. Therefore, ILIS are regularly used to correct for several compounds [15,40]. Nevertheless, the performance of each ILIS for correcting matrix effects need to be carefully evaluated. When appropriate ILIS are unavailable, matrix effects may be minimized by applying an additional clean-up step, but also lower pre-concentration factors may occasionally be desired for some substances in order to reduce ionization suppression and increase their detection limit [27,60]. In general, even when

ILIS are available, a reduction of matrix effects is recommended for better precision, sensitivity and robustness in complex matrix samples [60].

3. Chromatographic separation

Good chromatographic separation is important to reach the required levels of selectivity, sensitivity and identification power to monitor NPS through wastewater and pooled urine analysis. GC-MS has been applied for the determination of NPS in urine. However, because of the high levels of selectivity and sensitivity provided by this technique, it requires the derivatization of the analytes which results in a more time-consuming and less generic sample treatment [61,62]. Alternatively, LC-MS allows the determination of compounds with a broad range of polarity, low volatility and thermolability with the application of more generic sample treatment strategies. In addition, the aqueous nature of the matrices makes LC-MS fully compatible with the determination of NPS in wastewater and pooled urine samples [63].

Reverse-phase LC (RPLC) separates compounds within the range of low-polarity to non-polarity. Therefore, it seems to be the most suitable chromatographic technique to achieve generic and good chromatographic separation especially for wide-scope monitoring of NPS. Consequently, the vast majority of studies dealing with multi-residue methods in wastewater and/or pooled urine samples applied RPLC as the separation technique [13,17,25,64,65]. However, more polar (or ionic) substances such as amphetamine-like stimulants or synthetic cathinones and their metabolites, might require more specific methodologies. Recent developments in column chemistries and improvement in robustness of existing stationary phases allowed the analysis of more particular scenarios. Hydrophilic interaction LC (HILIC) is an alternative approach to effectively separate small and highly polar NPS. For example, Kinyua *et al.* [55] successfully developed a multi-residue methodology for the determination of 7 synthetic cathinones and amphetamine-like stimulants by means of HILIC separation.

Additionally, enantiomeric analysis has also been explored for the determination of NPS [66–68]. Chiral NPS are usually consumed as racemic mixtures of different forms (*i.e.* with an enantiomeric fraction (EF) between the two forms of approximately 0.5), even though both forms might differ quantitatively and qualitatively in the pharmacological activity [69]. Therefore, enrichment of the R

(or S) form, depending on the stereoselective metabolism in humans, is expected in biological samples [66]. Consequently, an EF found in wastewater or pooled urine samples deviated from the original EF value could help in distinguishing between human consumption and direct disposal of unused substances [66]. Other chromatographic techniques such as capillary chromatography and supercritical fluid chromatography (SFC) are promising strategies for the monitoring of NPS. The improvement in sensitivity provided by capillary chromatography, especially for the small amphetamine-like structures, revealed a technique to explore for this purpose [26]. Also, recent developments in commercially available instruments has seen an increase in applications of ultra-high performance (UHP) SFC - MS/MS, in particular using (sub)supercritical carbon dioxide (CO_2) with various organic additives as mobile phase [58,70]. One of the main advantages of UHPSFC compared to conventional UHPLC is its increased chromatographic efficiency and resolution [71] also permitting the separation of several NPS isomers with good results [72].

4. Quantitative target monitoring

As discussed above, the determination of NPS can be challenging due to the large number of potentially relevant compounds and the low concentrations expected in samples, in particular when considering wastewater and pooled urine. In fact, due to the often low prevalence of use of individual compounds, concentrations of these substances are often orders of magnitudes lower compared to conventional illicit drugs (<10 ng L^{-1}) [55]. For this reason, targeted methods, specifically using LC-MS/MS with QqQ or ion-trap mass analyzers, have been implemented for the reliable identification and quantification of selected NPS in urine and wastewater samples [15,17,73,74]. The development of such quantitative target methods, however, requires access to reference standards for precursor-product ion transition selection in the Selected Reaction Monitoring (SRM) mode and MS parameters optimization. Identification and confirmation is achieved through the acquisition of at least two SRM transitions and matching of the retention time (RT) and ion-intensity ratios between the sample and reference standard [75,76]. The most sensitive SRM transition is commonly selected for the quantification at low concentration levels, whereas the second transition allows confident confirmation [26,40,46]. However, since NPS often retain high structural similarity, the risk of selecting common transitions is present and therefore the acquisition of more transitions (if feasible) is recommended to gain more confidence to the confirmation process. Hence, it is also important to understand fragmentation of each NPS as it allows the selection of specific product ions and avoid non-specific transitions such as a neutral loss of water or CO_2 [77]. The latter is especially relevant to minimize potential matrix interferences when analyzing NPS at low concentrations in highly complex matrices such as pooled urine and raw wastewater samples. Although quantitative target monitoring can be performed using LC-HRMS instruments, their application in the field is limited due to the generally lower sensitivity compared to low resolution MS/MS instruments [17]. Hence, the advantage of low-resolution instruments for quantitative analysis lies in the robustness, selectivity and sensitivity which can be achieved by monitoring these specific precursor-product ion transitions. Combined with their high

scanning speed, these instruments can monitor many transitions almost simultaneously, and consequently high-throughput, multi-residue methods that include many targeted NPS biomarkers, can relatively easily be developed.

Synthetic cathinones, phenethylamines, tryptamines and piperazine-derivatives have been quantitatively determined in pooled urine samples collected during weekends at specific night settings [25] or at music festivals [23]. Although data obtained from quantitative determination of NPS in pooled urine samples only gives an indication on the extent of use for an NPS compared to other substances found in a specific sample [23], these findings are still very valuable, as the application of these selective and sensitive target quantitative methods give high confidence and allows confirmation of the NPS identified at low concentration levels. Synthetic cathinones are by far the most studied group of NPS in wastewater, followed by synthetic cannabinoids and phenethylamines. Studies using LC-MS/MS to monitor these substances have been carried out in Europe, Asia and Australia [15,17,26,39,40,78,79] and have shown spatial and temporal trends using population-normalized data. Although LC-MS/MS methods are highly sensitive and multi-residue methods can be developed, they have a major drawback, namely reference standard materials need to be available for method development as previously highlighted. Given the high number of NPS that have been detected in the market and their transient nature, reference standards are mostly available for only a limited number of compounds. Moreover, by the time reference standards become available, these compounds might have already disappeared from the market as they may have been less popular or added to the lists of regulated substances and can thus not be sold legally anymore. Further exacerbating the determination of these substances is the extent of their metabolism. There have been studies carried out on the metabolism of NPS using human liver microsome incubations to better understand the metabolism of certain NPS [80–84]. In addition, recent advances in computing power have permitted the development of comprehensive knowledge based software to predict the metabolic fate [85,86]. However, reference standards of most of the metabolites proposed are not commercially available and therefore unsuitable for quantitative target monitoring. Thus, quantitative target LC-MS/MS methods,

although indispensable to achieve the highest sensitivity needed for certain types of substances (*e.g.,* fentanyl and its derivatives), need to be complemented by other analytical approaches which allow a quick and broader monitoring, without the necessity for reference standards. Although low-resolution mass spectrometry (LRMS), especially tandem MS instruments, are highly appreciated in quantitative analysis, its application to qualitative analysis and capabilities in detecting unknowns is, limited due to the relative low resolving power (approximately 1 Da) and low sensitivity in full scan mode [77]. The use of HRMS offers new possibilities in the determination of NPS as well as circumventing some of the limitations of LRMS.

5. Qualitative screening approaches

HRMS presents strong potential for monitoring a large number of substances, due to its acquisition of accurate-mass full spectrum data at good sensitivity [63,77,87]. In order to facilitate the reading of this tutorial, terms that will be used in this section are defined below:

Target screening based on HRMS allows the qualitative screening of NPS after data acquisition based on large databases, thus evading the pre-selection of analytes for method development and the need of reference standards. However, the information included in the database is limited by the availability of reference standards. When reference standards are available, information such as accurate masses of fragment ions, adduct formation and RT can be included, whereas only the elemental composition, exact mass and theoretical isotopic pattern can be included when no reference standard is available. Although the acquisition of data is performed in an untargeted way, the approach is considered targeted and generally known as *suspect screening* [77,87], since the search is based on a list of target compounds that can be expected to be found in the samples. An advantage of this approach is that retrospective analysis can also be performed at any time from the acquired data to search for substances initially not considered and included in the database, such as novel NPS or newly discovered metabolites [88,89]. It should, however, be noted that the detection of some substances might be restricted by the sample treatment, the chromatographic conditions or the ionization efficiency [90], since usually a generic analysis is performed and no optimization has been executed for the NPS included in the database.

Non-targeted screening, without any selection of analytes, allows the investigation of any other NPS biomarker not included in the database. However, it implies an examination of each chromatographic peak and extensive investigation of its accurate mass spectrum. This process is challenging and time consuming and probably does not outweigh the rate of success in identifying of unknown NPS. Alternatively, the screening can be directed to discover related

compounds of known NPS using characteristic mass spectral information and applying mass-defect filtering or common fragmentation pathways.

As a starting point for researchers interested in undertaking qualitative screening of NPS by HRMS, the review article written by Hernandez *et al.* [63] describing different mass spectrometric strategies for the investigation of illicit drug biomarkers in wastewater is recommended. Although similar strategies and identification criteria can be applied for the investigation of NPS in pooled urine and wastewater, the challenges are different due to the rapid turnover in the NPS drug market creating a scenario with constantly moving analytical targets and the often lower prevalence of use compared to conventional illicit drugs. Moreover, the structural similarities of NPS and their metabolites often requires increased identification confidence in order to minimize reporting false positives. In the text below, practical examples are given to discuss different data acquisition workflows and data exploration approaches to illustrate how HRMS can help in the confident identification of NPS in high-complex pooled urine and wastewater samples.

5.1. Acquisition modes for hybrid high resolution mass spectrometric systems

The most commonly used HRMS analyzers are time-of-flight (TOF) and Orbitrap, which can be coupled with LC and possess high mass resolving power (>20,000 Full Width at Half Maximum (FWHM)) and mass accuracy (<5 ppm) for wide scope screening of NPS in pooled urine and wastewater [17,75,76]. However, hybrid configurations, such as quadrupole-TOF (QTOF) or quadrupole-Orbitrap (Q-Orbitrap), are nowadays more the standard than the exception as they considerably increase the potential of HRMS for screening NPS [20,21,27,44,52,91]. When working in MS/MS mode, it is possible to record accurate mass product-ion spectra of previously detected candidates and obtain relevant structural information to allow suspected NPS to be confidently identified or disregarded as false positives. However, the simultaneous accurate-mass acquisition of both full-spectrum and product-ion spectra data is preferable and collects accurate mass data of both the (de)protonated molecules

and its fragment ions in a single acquisition and without the selection of precursor ions.

In data-dependent acquisition (DDA) mode, the instrument first performs a "survey scan" from which the analyst chooses (or not) certain ions that fit specific criteria based on, for example, intensity thresholds. Ions for which these conditions are met, are then selected to be included in a list of preselected masses and fragmented to provide information-rich product ion scans. Unlike intensity thresholds, an inclusion (or exclusion) list allows large matrix interferences to be ignored, thereby facilitating the identification process and saving effort and time [27,52,63,92]. However, the size of the inclusion list (*i.e.*, suspects to be fragmented) can adversely affect the cycle time of the instrument. Therefore, a decrease in the number of scans (or data points) across a chromatographic peak will occur, reducing its detectability. Moreover, any compound not included in the initial inclusion list cannot later be retrospectively analyzed, so the sample would have to be re-extracted and re-analyzed. Yet, there is a way around this limitation, utilizing complementary targeted and untargeted DDA. This technique initially conducts an MS scan followed by targeted MS/MS using an inclusion list and then untargeted MS/MS on *n*-selected precursors. For example, analysts can look at MS/MS of the *n* most abundant precursor ions, which would be of great utility for samples with high levels of NPS such as seizure samples [14,93]. However, the generally low concentration of NPS found in pooled urine and wastewater might mask the detection of low abundant peaks, and therefore, many NPS may remain undetected [94].

Data independent acquisition (DIA) allows the acquisition of accurate-mass full-scan spectra under different collision induced dissociation conditions within a single injection. This acquisition mode is known under different names depending on the manufacturer (*e.g.* All-ion-fragmentation (AIF), all-ion MS/MS, MS^E and broadband collision-induced dissociation (bbCID)), where all ions generated in the ion source are sent to the collision cell for fragmentation without precursor ion selection or any predefined selection criteria. This alternation between full-scan and untargeted MS/MS events at low collision energy (LE) and high collision energy (HE), respectively, allows one to obtain

information relating to the accurate masses of the (de)protonated molecule as well as their fragment ions. Furthermore, it conserves highly valuable information on adducts and isotopes since the quadrupole works as an ion guide [63,77]. The main limitation of DIA is that spectra are non-selective and contain product ions for all ions formed in the ion source. Hence, the interpretation can be challenging, since co-eluting compounds or matrix interferences may "contaminate" the spectra, and makes it difficult to associate product ions with the correct (de)protonated molecule [14,95,96].

Slightly different modes compared to the other DIA modes mentioned above in terms of specificity have been developed by manufacturers with the objective to have HE spectra approaching to MS/MS quality data. As an example, in Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH) mode, a TOF MS full scan at LE is acquired, alternated by SWATH experiments at HE obtaining MS/MS data by fragmenting only the (de)protonated molecules present in a much narrower window (*e.g.* 15–25 *m/z*). In this way, SWATH can distinguish co-eluting compounds of different masses by having specific experimental mass fragmentation windows which filter out all masses not included in the specified mass range. This results in cleaner spectra, which facilitates identification [96,97]. This is a particular important point in the determination of NPS, which are notorious for the analytical challenges associated with common fragments. **Figure 1** shows the utility of SWATH in differentiating two co-eluting NPS, butyryl fentanyl with *m/z* 351.2431 and furanylfentanyl with *m/z* 375.2067 in a spiked wastewater sample. In the full scan acquisition at LE, it can be observed from the individual extraction ion chromatograms (XICs) that the two NPS seemingly elute at 12.50 min (**Figure 1A, top**), with the mass spectra at this RT showing both masses (**Figure 1A, bottom**). However, when applying SWATH, the HE experiments carried out at different mass windows (*m/z* 340.2–357.4; **Figure 1B** and *m/z* 372.6–389.8; **Figure 1C**) allowed them to be distinguished by extracting the mass of each of these fentanyl derivatives in their corresponding acquisition window. With the mass of butyryl fentanyl and furanylfentanyl falling within separate experiments, they can be individually extracted and identified using cleaner

spectra. This exemplifies the power of this acquisition mode in the elucidation of NPS.

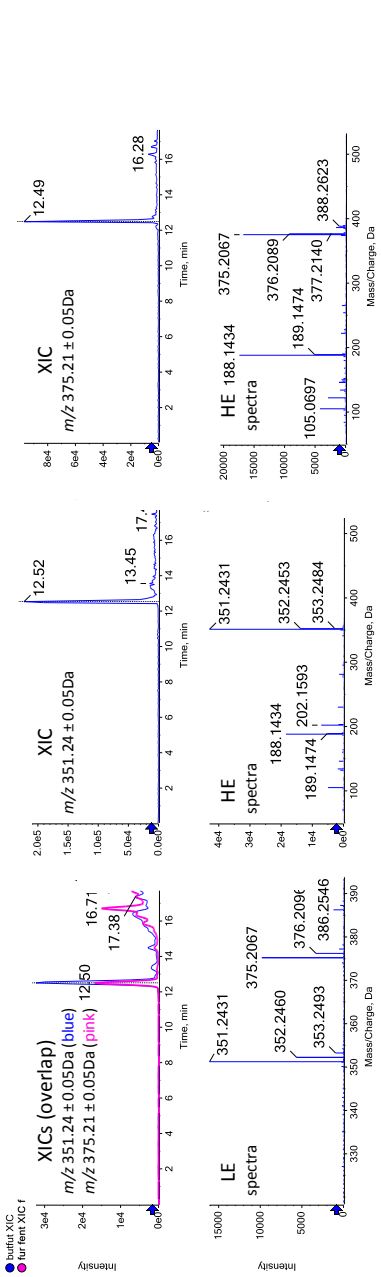


Figure. 1. Identification of two co-eluting NPS, butyryl fentanyl (*m/z* 351.2431) and furanylfentanyl (*m/z* 375.2067) in a spiked wastewater sample using Sequential Window Acquisition of all THeoretical fragment ion spectra (SWATH). (A) Overlapping extraction ion chromatograms (XICs) of the two NPS with chromatographic peaks eluting at 12.50 min (top); full scan acquisition mass spectra with low collision energy (LE) (10 V) at retention time 12.50 ± 0.10 min (bottom). (B) SWATH mass window *m/z* 340.2–357.4, XIC at *m/z* 351.24 (middle) and high collision energy (HE) mass spectra (bottom); (C) SWATH mass window *m/z* 372.6–389.8, XIC at *m/z* 375.21 (middle) and HE mass spectra (bottom).

5.2. Suspect screening

Suspect screening approaches usually take advantage of home-made databases. However, the information included therein is limited by the availability of reference standards, as previously explained. When no reference standard is available, the minimum suggested requirements for a tentative identification is the accurate mass of the (de)protonated molecule and, at least, one significant fragment ion together with the corresponding isotopic pattern. This is in the line with proposed quality procedures recommended in other research fields [76,98]. The observed fragments need to be in accordance with the chemical structure and, preferably, in agreement with previously reported data in scientific literature or online spectral databases [27,52,99,100]. Ideally, reference standards are available, and information such as accurate masses of fragment ions, adduct formation and RT can be included, which allow unequivocal identification. However, this entails high costs due to the high number of compounds and, therefore, huge efforts have been devoted, in the recent years, to develop community-made or online mass spectral databases for NPS. The best known databases are NPS Data Hub [101] and HighResNPS [102,103] with more than 2800 and 3350 entries, respectively (date accessed: June 26, 2020). The HighResNPS library currently has active users from more than 10 laboratories around the world with the intention to ensure up-to-date analytical information from the moment a specific NPS becomes available to a given participating laboratory [102]. These libraries are available to help and facilitate the screening of NPS and their metabolites [101,104–106].

In most laboratories, a suspect screening based on large home-made databases is often the first step for monitoring samples. Due to the high number of NPS and metabolites, the rapid transience of these compounds on the market, high costs and limited availability of reference standards, home-made databases are normally built of merely accurate masses of the (de)protonated NPS and fragment ions. Yet, the low concentration levels of NPS present in combination with strong matrix interferences makes the tentative identification of NPS challenging and remark often the necessity to perform some additional research or experiments to increase the confidence in the tentative identification. As an

example, **Figure 2** shows the tentative identification of 4-chloro-α-pyrrolidinopropiophenone (4-chloro-α-PPP) in a pooled urine sample. Its protonated molecule, the isotopic information related to the presence of one chlorine atom and at least one fragment ion was observed at accurate mass (**Figure 2A**). However, a known and abundant fragment of 4-chloro-α-PPP at *m/z* 167.0258 [107] showed an undue high mass error (+143 ppm) under the initial screening conditions, which made the tentative identification of this NPS questionable. By increasing the mass resolution of the Orbitrap MS from 20.000 to 35.000 FWHM and zooming in the *m/z* range of the fragment, it was possible to distinguish three peaks at *m/z* 167, one corresponding to the fragment ion *m/z* 167.0258 (+5.3 ppm) of 4-chloro-α-PPP (**Figure 2B, bottom**). This allowed more confidence to be gained in the identification. Subsequently, the feature could be identified as 4-chloro-α-PPP by means of a reference standard. The latter is pivotal for the confirmation of the identity of the NPS. However, by using this approach, laboratories do not need to purchase all reference standards a priori to the analysis [108] and could prioritize those NPS for which more reliable evidence is obtained.

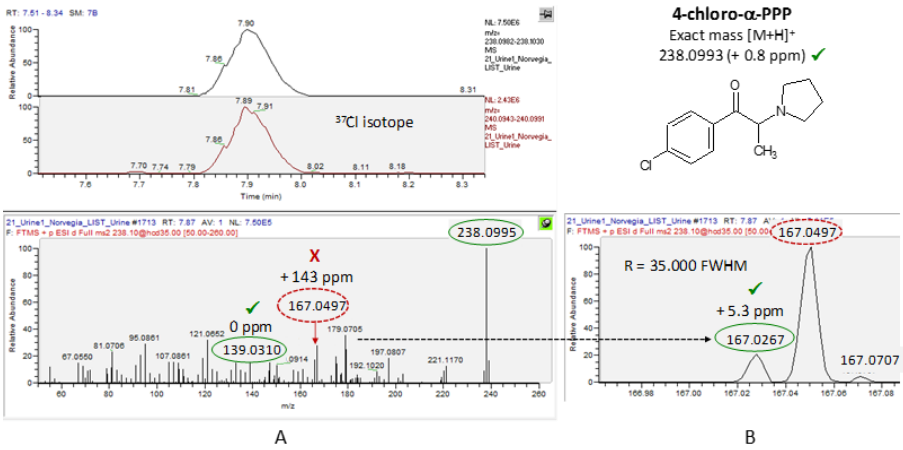


Figure 2. Tentative identification of 4′-chloro-α-pyrrolidinopropiophenone (4-chloro-α-PPP) in a pooled urine sample. (A) Extracted ion chromatogram of 4-chloro-α-PPP and ^{37}Cl isotope (top); Product ion mass spectra of $[M+H]^+$ at *m/z* 238.10 (bottom). (B) Structure of 4-chloro-α-PPP (top); Zoom in the range of fragment ion with *m/z* 167 at resolution (R) of 35,000 Full Width at Half Maximum (FWHM) (bottom).

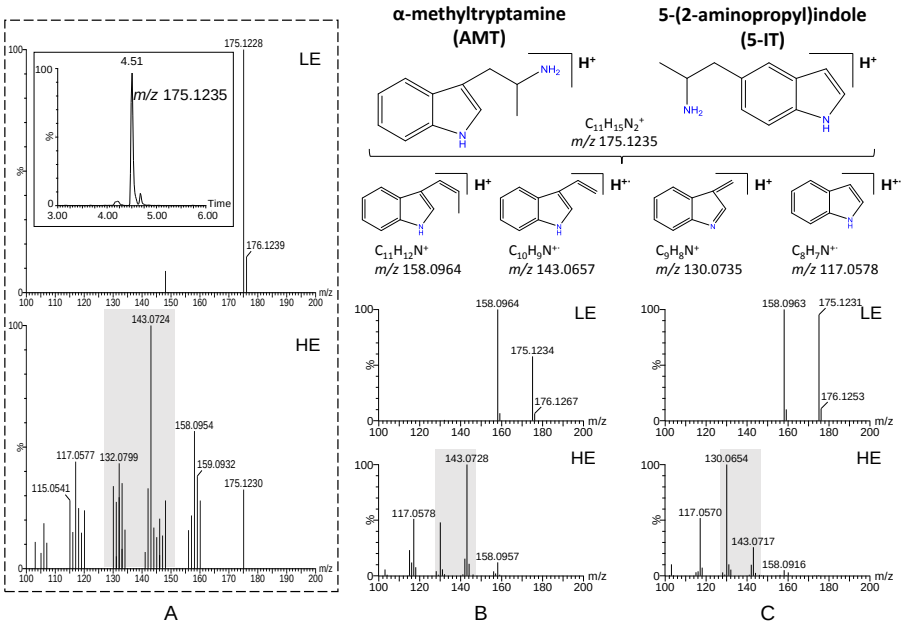


Figure 3. Identification of α-methyltryptamine in a raw wastewater sample using QTOF MS. (A) Feature detection of *m/z* 175.1235 at 4.51 min (top, insert) together with the low collision energy (LE) spectra (top) and high collision energy (HE) spectra with emphasis on *m/z* 130–145 (grey areas) (bottom); (B) Structure, fragment ions, LE and HE spectra of α-methyltryptamine; (C) Structure, fragment ions, LE and HE spectra of 5-(2-aminopropyl)indole.

Positional isomers or homologues are frequently the first choice to substitute banned NPS [109]. Hence, NPS often have only minor modifications to a backbone structure and the structural similarities of NPS and their metabolites are often reflected by their common fragmentation pathways, this poses one of the principal challenges in suspect screening strategies. As an example, the analysis of a raw wastewater sample showed a chromatographic peak at 4.51 min giving a positive hit for the isomers α-methyltryptamine (AMT) and 5-(2-aminopropyl)indole (5-IT) based on the accurate mass of their protonated molecule and their fragment ions (**Figure 3A**). These two isomers share the same chemical backbone with the only difference being the position of the substituent (**Figure 3B and C, top**). The following MS fragment ions were found:

m/z 158.0954, *m/z* 143.0724, *m/z* 132.0799, *m/z* 117.0577 and *m/z* 115.0541, with the most abundant fragment at *m/z* 143.0724 (**Figure 3A, bottom**). The only difference, described in the literature, between the spectra of AMT and 5-IT resides in the relative intensities of the fragment ions [110]. The most intense fragment ion of 5-IT has an *m/z* of 130, whereas the most abundant fragment ion for AMT corresponds to *m/z* 143. This slight difference in the fragmentation pattern (*i.e.* intensities) gave more confidence in the tentative identification of AMT instead of 5-IT in this sample. Therefore, AMT was synthesized and a reference standard of 5-IT was donated by a collaborating laboratory. When comparing that empirical data to AMT and 5-IT reference standard MS fragment ions (**Figure 3B and C, bottom**), it can be observed that both substances share the same fragment ions (in nominal mass; *m/z* 143, *m/z* 130, *m/z* 117 and *m/z* 115) coinciding with the fragment ions observed in the sample, but that AMT indeed show a more abundant fragment ion with *m/z* 143. This gave more confidence in the positive identification of this NPS and together with its RT, AMT could finally be confirmed.

5.3. *In-silico* approaches

In some cases, the instrument-specific parameters (*i.e.* accurate mass ions and isotopic patterns) do not suffice to tentatively propose a chemical structure, and, therefore, additional studies are required. For that purpose, predictive models have been used to filter out false positives and increase the confidence of compound identification when reference standards are unavailable or no information is within reach in previously reported data [27,111,112]. Aalizadeh *et al.* developed a RT prediction model using Quantitative Structure-Retention Relationships (QSSR) and Support Vector Machines (SVM) to model the RT data for both HILIC and RPLC with high accuracy [111]. A different approach was proposed by Bade *et al.* considering the application of Artificial Neural Networks (ANNs) for the development of a RT predictor for gradient-RPLC using a dataset of more than 500 compounds with a predictor accuracy of ±2 min [112]. Such RT predictive tools are highly valuable for the determination of NPS in complex matrices as demonstrated by Diamanti *et al.* [27]. Since the availability of reference standards is limited, the suspect screening of NPS usually results in

many candidate structures because of the structural similarity of many NPS, as for example, in the case of the two isomeric phenethylamines 3,4-methylenedioxy-*N*-hydroxyethylamphetamine (MDHOET) and *N*-hydroxy-*N*-methyl-3,4-ethylenedioxyamphetamine (EFLEA). The predicted RT using a QSSR predictor model matched the one for MDHOET and discarded the one for EFLEA, thereby reducing the number of candidates and increasing the confidence in the tentative identification of MDHOET in influent wastewater from Athens [27]. *In-silico* fragmentation tools, such as the MetFrag software, are pivotal in a suspect screening workflow. This software generates a predicted fragmentation of molecules based on their structure and compare it to the empirical data gathered proposing a list of fitting candidates together with a scoring parameter [113,114]. However, it is common that many structurally related substances can be assigned to the empirical data with a similar score value [113], which is a drawback particularly for the investigation of NPS because of the similarity of several substances.

6. Ion mobility separation coupled to high resolution mass spectrometry

The recent development of the hyphenation of IMS with LC-QTOF MS instruments (LC-IMS-QTOF MS) represents an innovative tool for their application in target and non-targeted screening strategies. IMS separates ions depending on their size, shape and charge in a gas phase, (usually nitrogen or helium), and in the presence of an electric field [115]. Ion separation occurs in the millisecond time scale, making it compatible with fast TOF MS acquisitions [116]. The time an ion takes to travel through the mobility cell *i.e.* the drift time (DT), adds an extra dimension to the obtained chromatographic RT and accurate mass, which results in increased selectivity and improved identification, particularly in DIA modes [116,117]. The increased selectivity is translated into much cleaner and higher-quality spectra than conventional HRMS DIA spectra, since (de)protonated molecules and fragment ions of interest with the same DT can be aligned and separated from co-eluting matrix components. Although data sets inherently become more complex and more comprehensive, the utilization of IMS-HRMS instruments does not overcomplicate the data revision process thanks to the four-dimensional automatic feature detection. This allows the software to both deconvolute peaks based on chromatographic and MS data and align ions with the same RT and DT into unique features. Thus, LE and HE spectra are DT filtered for the deconvoluted ions (*i.e.* for each ion detected in the LE spectra its DT is used to correlate it with the fragment ions obtained in the HE spectra). Cleaner spectra can also be obtained by improving the chromatographic separation. Although improvements in the quality of the spectra often relies on spectral discrimination of the compounds, a good chromatographic separation is recommended especially when analyzing complex matrices such as pooled urine and wastewater that contain many co-eluting interferences. Yet, IMS provides an extra dimension of separation which fits between chromatography and MS and results in cleaner spectra, but without increasing the chromatographic run time or mass resolving power.

A further advantage of IMS is that Collision Cross Section (CCS) values can be derived from the DT and represent the surface of the sphere created by the ion

when moving in the gas phase. Unlike DT, CCS is an instrument independent value, provided that the same drift gas and ion mobility calibration standards are used [116,118,119]. The importance of CCS values relies on the fact that they are robust across multiple platforms (*i.e.* deviation up to 2%), independent of the chromatographic conditions used and not affected by matrix composition [118–120]. CCS values depend on the calibration procedure applied, and the deviation between instruments is caused by the slight experimental variations in room temperature, gas pressures and other hardware settings. Hence, CCS is a parameter that can give support to MS-based compound identification in addition to RT, *m/z*, isotopic pattern and fragment ions. Finally, IMS enables, in theory, the separation of isomeric compounds not previously resolved using LC, since they are expected to have a different mobility in the drift cell, and therefore different CCS values [121,122]. Although there is a relationship between the *m/z* and CCS, Bijlsma *et al.* [123] showed that a range of 35 $Å^2$ could be observed for molecules of approximately 300 Da, therefore, demonstrating that no direct correlation between *m/z* and CCS could be established and that thus IMS may separate isomers.

Figure 4 illustrates the benefits of IMS in terms of higher-quality spectra in DIA MS/MS events. In this example, a positive finding of ketamine in a wastewater sample is shown using an ion mobility separation QTOF MS (Vion from Waters). When searching for ketamine (with *m/z* 238.0993 ≤ 5 ppm) a chromatographic peak at a RT of 3.33 min was observed (**Figure 4A, top** (yellow arrow)). The corresponding conventional DIA MS^E spectra (LE and HE) show many ions when no DT alignment is applied (**Figure 4B, top**) resulting in a base peak with *m/z* 263.1386, which does not correspond to ketamine (*i.e. m/z* 238.0993, highlighted in green). However, when applying the IMS MS^E acquisition mode ($HDMS^E$, High-Definition MS^E), several co-eluting ions at 3.33 min are separated in the mobility cell, illustrated as red or black dots in **Figure 4A, bottom**. The DT of the ion with *m/z* 238.0993 was 4.89 ± 0.20 ms and the corresponding fragment ions in this range, the blue highlighted areas, can be aligned. All other ions outside this area are filtered out, which results in much cleaner and easier to interpret spectra (**Figure 4B, bottom**). Despite the presence of some co-

eluting interferences with similar DT, the resulting spectra contains fragment ions which could be primarily assigned to ketamine [124].

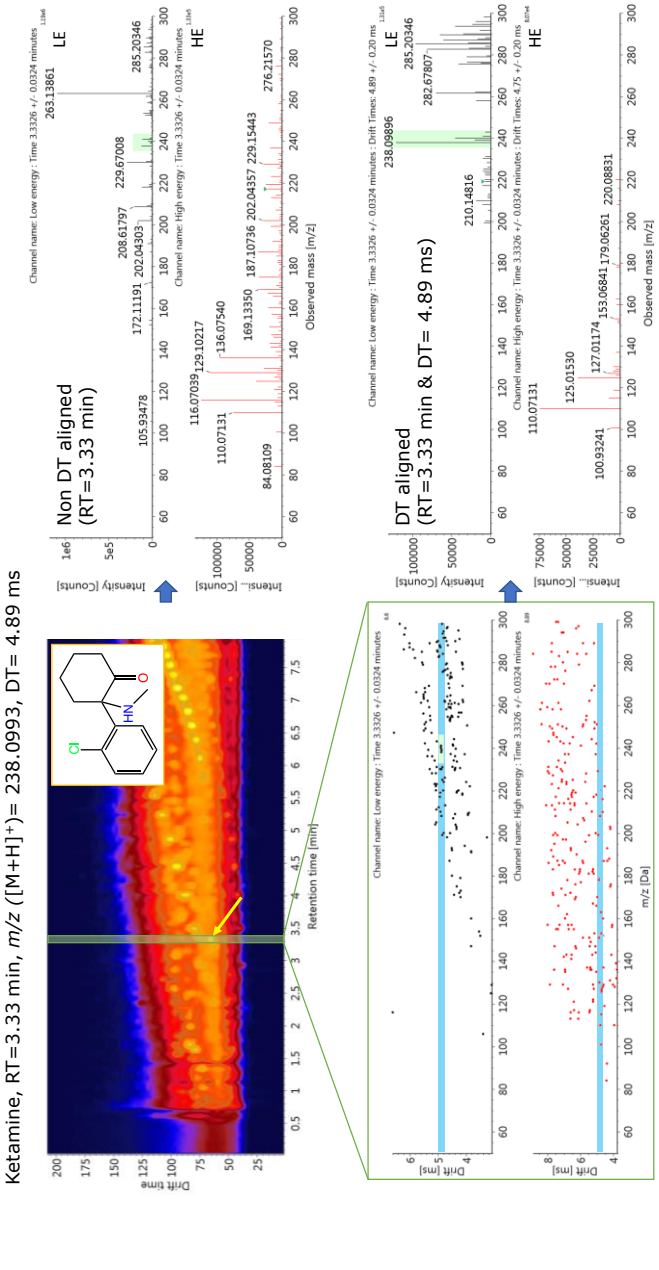


Figure 4. Identification of ketamine in a raw wastewater sample using IMS QTOF MS. (A) Feature detection of *m/z* 238.0993 at 3.33 min and drift time (DT) 4.89 ms, yellow arrow (top) (*70 µs/scan); co-eluting ions at 3.33 min illustrated as red or black dots and separated by DT. Blue highlighted areas are the DT ranges of 4.89 ± 0.20 ms at *m/z* 238.0993 at low collision energy (LE) and high collision energy (HE) (bottom). (B) LE and HE mass spectra without IMS DT alignment (top); LE and HE mass spectra with IMS DT alignment (bottom).

The additional cleaning of spectra provided by IMS is of particular relevance for the determination of NPS in challenging matrices such as wastewater or pooled urine where thousands of naturally occurring compounds can hamper the identification of these substances at the low concentration levels expected. Moreover, since the CCS value of a certain molecule is not affected by matrix composition, their utilization as an additional identification point in the determination of NPS pushes IMS-HRMS as a promising technique in the monitoring of these substances [125,126]. Therefore, the development of home-made or collaborative on-line databases including ion mobility data will enhance the efficiency of target NPS screening. However, as has been discussed earlier, due to the lack of analytical standards for most of the NPS and metabolites and the still sparse accessibility to IMS-HRMS instruments in research centers, the availability of CCS values for these substances is still very limited. Hence, *in-silico* predictive tools similar to those for RT and MS fragmentation may help to increase the confidence in the identification of tentative candidates. Several data-driven CCS predictor systems have been developed for the prediction of CCS values for small molecules [123], pharmaceuticals and drugs of abuse [127] and metabolites [128]. As an example, the predictor reported by Bijlsma and Bade *et al.* [123] was developed using 205 CCS values for small molecules including pharmaceuticals, pesticides and drugs of abuse with ANNs for modelling the ion mobility data. Although the empirical variability of CCS measurements across instruments for a certain molecule is known to be up to 2%, with the developed CCS predictive model, the maximum deviation at the 95% confidence interval was only 6%. Mollerup *et al.* [127] were able to reduce the deviation in the predicted CCS to a 4%, consequently increasing the accuracy of the model. In the case of the predictor model developed by Zhou *et al.* [128], support vector regression was applied to the development of predictive models for different molecular adducts with median relative errors of approximately 3%. Regardless of the predictive model applied for the prediction of CCS, the utilization of these strategies facilitates the tentative identification of NPS in suspect screening strategies [125], especially when combined with RT and MS fragmentation predictive tools.

7. Future perspectives

The determination of NPS in pooled urine and urban wastewater has shown several challenges due to distinct factors as discussed in this manuscript. Current analytical instrumentation based on LC combined with LRMS and HRMS and the application of complementary data acquisition workflows and data exploration approaches helps to circumvent or confront certain barriers. However, more research related to NPS biomarkers is required and several trends in analytical chemistry, which is under continuous development, can be highlighted:

i. ***NPS biomarker selection.*** The high number of existing NPS and the constant introduction of new compounds on the drug market creates a dynamic scenario of moving target biomarkers. Hence, monitoring of all NPS is complex and efforts could therefore be initially focused on NPS which are relatively high-dosed or frequently consumed and excreted (partly) unchanged such as amphetamine-like substances and cathinones. Especially since scant information on NPS pharmacokinetics is currently available, which complicates the choice of suitable biomarkers (parent substance or urinary metabolites) [129,130]. This is particularly relevant for synthetic cannabinoids and compounds like NBOMes that are highly metabolized in the human body [42,131,132] and for synthetic opioids that are consumed at very low doses [39], leading in both cases to very low concentration levels of the corresponding biomarkers in urine and, consequently, in wastewater. However, there are some published works on the metabolism of NPS [80–84] and different computational tools exist that predicts the metabolic fate of chemicals [86,87]. Although the proposed metabolites therein are generally not commercially available for quantitative target monitoring, these compounds should be included within screening databases as well as aiding in retrospective data analysis to ensure that the most appropriate analytical targets are investigated.

ii. ***Sample collection, storage and treatment*** of pooled urine and wastewater is pivotal for getting meaningful information on NPS use. Pooled urine analysis of samples collected from portable toilets and

urinals give an informative snapshot of the NPS used, but is often limited to men only and it is difficult to extrapolate results to the total number of toilet users. All-gender toilets with an improved design, complying specific technical requirements like a flushing mechanism and a visitor counter could circumvent these limitations in future studies. Currently, daily composite wastewater samples are more representative and analysis provides population-normalized quantitative information on NPS. A best practice protocol to collect representative wastewater samples of an entire community is available [32] to ensure the comparability of results from different countries. However, wastewater is more diluted compared to pooled urine resulting in lower concentrations, which may complicate the detection of some NPS. Passive sampling increases the possibility to detect substances with low prevalence of use, because of the sampling and concentration of analytes over a longer period of time. Yet, passive sampling also merely gives a snapshot and has several limitations that need to be overcome or optimized as previously described. Recent developments, using diffusive gradients in thin films which, in contrast to conventional samplers, consist of a diffusive and binding gel and are exposed to the medium, are less dependent to hydrodynamic condition (*e.g.* flow rates) and can hence overcome some of the limitations encountered with conventional passive samplers [133,134].

A relevant requirement for an NPS biomarker is its stability in pooled urine and wastewater in order to avoid any loss that can prevent detecting its use. Further work need to be addressed to test biomarkers stability and potential degradation or transformation in raw wastewater and urine [39–41,94]. Until more information is available, it is recommendable to store samples in the dark at −20 °C directly after sample collection in order to minimize possible degradation.

Sample treatment is very important to improve detection. However, a versatile sample treatment to retaining a wide range of NPS is not always feasible and specific treatments for certain NPS classes such as

synthetic cannabinoids and synthetic opioids (*i.e.* high potency NPS such as fentanyl) need to be developed.

iii. Good ***chromatographic separation*** might seem less important when coupled to highly sensitive and selective mass spectrometers, although it can be essential in the detection and identification of NPS. Taking into account the many isomers or structurally related compounds and the often strong matrix effects, more effort could be put into chromatographic separation in future work. HILIC and enantiomeric analysis have demonstrated a strong potential to move a step forward into a more comprehensive determination of NPS in wastewater and pooled urine. Capillary chromatography and UHPSFC-MS/MS have also been explored. Yet, some concerns have also been raised related to the robustness of the technique to routinely analyze complex matrices. Future developments in terms of more robust column chemistries will open a new scenario for the monitoring of NPS. Additionally, UHPSFC has the potential to combine the advantages of LC and GC, thus improving analytical capabilities of laboratories dealing with the determination of NPS.

iv. Highly sensitive ***targeted methodologies*** based on LRMS will continue to play an important role in monitoring NPS use, particularly for those compounds which have established a niche market and/or are highly potent and require low detection limits. In addition, complementary **suspect screening approaches** based on large home-made databases, including many substances for which reference standards are not available, will remain the common practice for the foreseeable future. Furthermore, the improved sensitivity and quantitative capabilities of HRMS instruments combined to multi-stage off-line or on-line solid-phase extraction allow achieving targeted quantitative and qualitative screening analyses in a single run, thus overcoming the need of having two distinct instruments/methods [27]. Similarly, machine learning algorithms used to relate peak area of features recorded in HRMS analyses, chromatographic and mass spectrometric conditions to concentrations, might overcome the need for reference standards to

obtain an (indicative) information about analyte concentrations in measured samples [135]. Qualitative information about the presence or absence of given NPS in wastewater is informative and studies have shown some spatial and temporal trends [23,27,136], but only quantitative data can provide absolute comparisons by showing changes in community prevalence through concentrations or mass loads.

v. ***Non-target screening*** remains predominantly unexplored for the identification of NPS in pooled urine or wastewater. A genuine non-target screening without any selection of analytes to be searched is a very challenging and time consuming process and a more successful strategy would be the application of non-target screening directed towards the discovery of compounds structurally related to known NPS. In this case, the higher concentrations generally present in pooled urine makes this matrix most interesting for this approach. The expected improvements for the forthcoming years in the mass-resolving power of HRMS instruments in combination with higher scan-speed will allow the acquisition at higher mass resolution with more efficient chromatography. This development in instrumentation will improve sensitivity and can also be very useful to differentiate between isobaric compounds (*i.e.* compounds with the same nominal mass but different chemical formula and thus different exact mass). Moreover, improved mass resolving power does not only improve the separation of parent compounds, but can also help finding characteristic fragment ions and gain confidence in the obtain identification. Furthermore, improvements in software tools for peak picking and data deconvolution (*i.e.* the capability to find chromatographic peaks of compounds and to obtain high quality spectra) will aid to a successful identification of NPS, but the knowledge of basic rules in mass fragmentation and thus the expertise of the mass spectrometrists should not be overlooked in both suspect and non-target screening.

vi. The rapid transience of NPS in the drug market as well as the limited availability of reference standards for both NPS and known metabolites

poses an analytical challenge for the full confirmation of substances detected. Therefore, the development and continuous updating of ***collaborative and public NPS mass spectral databases*** will smooth the identification process since contributors and users to those databases will have access to empirical information without the need of having the reference standards in their own laboratories. Hence, the number of false positive identification (based on suspect and non-target screening) will be reduced since tentative identifications will be supported by empirical data from other researchers.

vii. As is the case with online databases, ***prediction tools*** ease the tentative identification of NPS. The development of metabolic, RT and CCS predictive models represent a turning point in the investigation of NPS. The continual development of more accurate and refined predictive models will make prediction tools even more powerful for the application of NPS consumption – particularly the complexity associated with structural similarities among NPS families. The small differences in the chemical backbone for most NPS classes and consequently similar physicochemical properties often make the current predictive tools less than ideal due to the analogous outcome obtained from the prediction.

viii. ***Retrospective analysis*** will continue to play an important role in uncovering trends in NPS consumption. HRMS analyses allow analysts to continually explore samples, without the time expense associate with re-extracting and re-analyzing samples. Reprocessing samples should be performed periodically, which can be a laborious task. Nevertheless, it is an interesting tool, as 'new' NPS and metabolites are found, standards become more available and predictive techniques become more commonplace, retrospective analyses can be performed to better reveal community use of NPS.

ix. ***Ion mobility separation*** coupled to HRMS has arisen as a useful technique and it is expected that it will gain in popularity. The cleaner and higher-quality mass spectra as well as the increased sensitivity of the instruments facilitates the identification process of NPS at low concentration levels and in complex wastewater or pooled urine

samples. Future improvements will be related to the resolution of IMS instrument to enhance the separation of isobaric or isomeric substances that cannot be previously resolved by chromatography.

8. Conclusions

Comprehensive analytical strategies can be applied to investigate NPS in pooled urine and wastewater, from quantification of target biomarkers to the detection and (tentative) identification of new substances and metabolites. The investigation of NPS in pooled urine and wastewater is a subject of current interest because, integrated with additional epidemiological information, it can be a useful tool for a comprehensive assessment of NPS use. In this context, data triangulation with traditional indicators, such as public surveys, online forums, data of drug testing services, police seizures and forensic analyses, is pivotal to gauge community consumption. Thus, the analysis of pooled urine and wastewater can complement other data and provide a more complete picture of community consumption.

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5.3. Discussion

International organizations such as EMCDDA and the UNODC set up strategies for the early detection of new substances to be controlled afterwards (EMCDDA, 2015, 2007; UNODC, 2017). The development of effective and successful drug policies relies on the availability of timely and accurate data on the drug scenario. These programs generally gather information through population surveys, seizures and/or intoxications from hospital emergency rooms. However, data provided by these sources of information may be biased by the level of toxicity of NPS consumed with not all NPS consumers ending up in emergency rooms, or due to the fact that consumers do not often know what they are really consuming (Reid and Thomas, 2016). Yet, collecting and modelling these data is time-consuming and, in the end, it might barely represent what is the reality of NPS market. Contrarily, wastewater analysis may provide a comprehensive, anonymous and almost real-time overview on the situation of NPS use within a community. Therefore, it is of additional help for public health agencies to compliment conventional sources of information considered that understating the extent and actual use of NPS within a community is an arduous task for decisionmakers and agencies.

Wastewater-based epidemiology has demonstrated its large potential to monitor psychoactive substances consumption within a community through the yearly monitoring campaign on ID consumption undertaken by the SCORE network (González-Mariño et al., 2020; Ort et al., 2014). Over the recent years, there has also been an effort within the scientific community to include NPS in monitoring campaigns (Bade et al., 2019a, 2021; Castrignanò et al., 2016; González-Mariño et al., 2016b, 2016a) with different analytical workflows to give insight about NPS consumption through WW analysis.

The EMCDDA has reported > 830 NPS since 2008 (EMCDDA, 2021) illustrating the versatility and rapid transience of the NPS market. Therefore, the international project 'NPS-Euronet. Identification and assessment of new psychoactive substances: A European network' (HOME/2014/JDRUG/AG/DRUG/7086, co-funded by the European Commission) aimed to combine targeted and untargeted analytical strategies to produce reliable data about the actual extent of NPS consumption in Europe. **Scientific articles 8, 9** and **10**, were developed under the framework of this international project in collaboration with different partners.

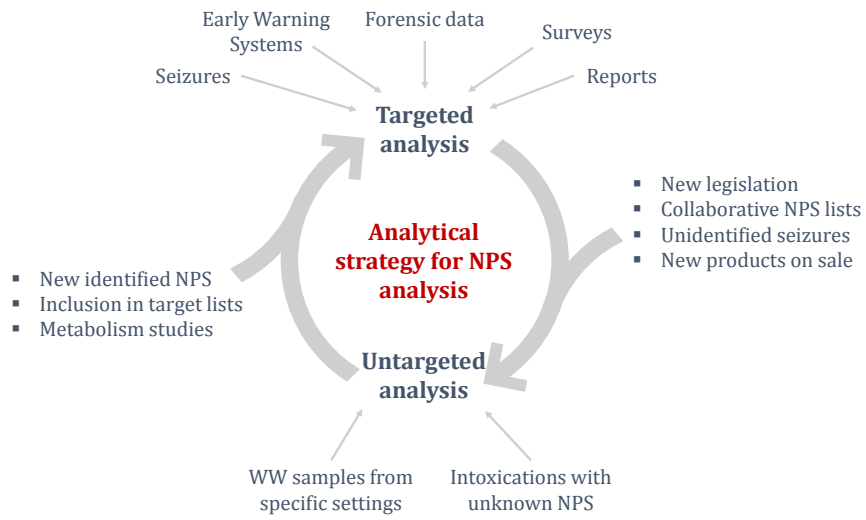


Figure 5.3. General overview of the analytical strategy for the monitoring of NPS substances through WW analysis.

In general, the overall analytical strategy to detect new NPS being consumed within a community should be a combination of complementary sources of information and analytical strategies (**Figure 5.3**). Information gathered through seizures, surveys, EWS, forensic data and public organism reports enable performing targeted analysis of NPS in wastewater samples by creating list of compounds of special interest. In addition, the finding of unidentified substances in seizures as well as the release of new products to the NPS market requires untargeted analysis of NPS. In this sense, smart collection of samples in specific settings such as night clubs or music festivals together with samples from hospital emergency rooms will increase the likelihood of detecting previously unknown NPS or metabolites or compounds reported in collaborative databases. Those results obtained from untargeted analysis may reveal the existence of new substances, either NPS or metabolites, to be monitored. Therefore, these new compounds need to be included in the target lists alongside with metabolism data to enable a comprehensive and constant maintenance of updated NPS databases. As shown, the monitoring of NPS through WW analysis requires a carefully throughout strategy with several aspects to be considered.

Some of the main key topics for a successful strategy can be summarized as follows:

i. ***Appropriate selection of NPS biomarkers.*** Selecting appropriate biomarkers for NPS consumption is pivotal to produce reliable data. Due to the high number of substances being constantly released to the market, the low availability of NPS reference standards and the consequently lack of metabolism studies for the vast majority of NPS, the establishment of appropriate biomarkers of consumption (either parent compound or urinary metabolite) is challenging (Gracia-Lor et al., 2017). Although little is known about the pharmacokinetics of most NPS, some studies have been published in this regard (Diao and Huestis, 2019; Fabregat-Safont et al., 2020; Lai et al., 2015; Mardal et al., 2016; Vervliet et al., 2019). For example, synthetic cathinones are a group of chemicals with moderate polarity that are scarcely metabolized when consumed and, thus, the parent compound itself can be monitored to

indicate consumption (Couto et al., 2018; González-Mariño et al., 2016b; Joshi et al., 2014). On the contrary, synthetic cannabinoids chemical structures show little polarity and are therefore highly metabolized in the human body leading to very low levels of parent compounds in urine samples and high levels of specific metabolites. Thus, the latter are the most appropriate biomarker for these substances (Bijlsma et al., 2018; Reid et al., 2014). However, the existence of reference standards for metabolites is even more reduced than for parent compounds and therefore only suspect screening strategies can be performed to monitor consumption of these compounds.

ii. ***Sample collection, storage and treatment.*** Obtaining meaningful information is essential for the understanding of the NPS consumption scenario. Therefore, sample collection, storage and treatment need to be well-planned. Collection of 24-h composite samples, as proposed for the analysis of ID by the SCORE network (Ort et al., 2018, 2010), seems a good strategy for NPS especially when more information is available. Meta-data associated with the sample such as influent WW flow rate at the sampling location and population served by the WWTP allows to calculate population-normalized data and, therefore, the comparison among different communities. However, back-calculation to consumption (mg day^{-1} 1000 inh^{-1}) is still not possible for most NPS mainly due to the lack of metabolism studies. In addition, and due to the low rate of consumption of NPS, sampling WW and PU at specific settings with expected higher consumption such as night clubs or music festivals (Archer et al., 2014, 2013; Bijlsma et al., 2020) will increase the likelihood of detecting new NPS being consumed. Also, the obtention of reliable data relies on the in-sample stability of biomarkers during transport and storage (Gracia-Lor et al., 2017). In this sense, appropriate storing conditions *i.e.* -20 °C and acidification to stop bacterial activity as well as short storage time are highly recommended (Bade et al., 2017; González-Mariño et al., 2016a; Senta et al., 2015). After storage, sample treatment is one of the main aspects to consider for the analysis of NPS. Solid-phase extraction to pre-concentrate

compounds of interest in WW and PU samples as well as to remove matrix endogenous chemicals is the preferred sample preparation approach to analyze NPS due to the increased sensitivity provided. However, due to the huge variety of compounds, no generic procedure is available to extract all existent NPS at once. Therefore, specific strategies focusing on different chemical families have been developed to ensure appropriate analysis for those substances being monitored (Bade et al., 2020a; Diao and Huestis, 2019; González-Mariño et al., 2016b, 2018b; O'Rourke and Subedi, 2020; Pandopulos et al., 2020).

iii. ***Population size estimation.*** Estimating population size is one of the biggest challenges and main sources of uncertainty for WBE applications. Although population census may well reflect the actual size of a city and it has been traditionally used for WBE, it might not denote an efficient and real time measurement of the amount of people contributing to the wastewater collected in the WWTP (Castiglioni et al., 2013). In this regard, several efforts have been made to establish improved population estimations by analyzing artificial sweeteners, caffeine, nicotine or even anthropogenic-endogenous compounds (Gracia-Lor et al., 2017). More recently, Thomas *et al.* showed how the utilization of mobile device data can better determine the dynamics of the population and, therefore, establish more accurate values for population size estimation in WBE applications (Thomas et al., 2017). However, at the moment of writing this thesis, no consensus has been reached on the population biomarker to be used and, population census is the recommended choice for data normalization.

iv. ***Control of direct discharges.*** Due to the lack of metabolism data or to the fact that the parent compound itself might be an appropriate biomarker for NPS consumption, parent compounds are often monitored in WW analysis. Therefore, direct discharges of NPS into the sewage system may distort the results provided by WW analysis. The discrimination between actual consumption or disposal poses an analytical challenge with not a straightforward solution. In cases in which metabolites are being monitored like synthetic cannabinoids, the

determination of the parent compounds at an elevated relative intensity compared to their corresponding metabolites might indicate direct disposals of unconsumed substances (Gracia-Lor et al., 2017). However, for those substances for which the parent compound is monitored, chiral analysis might shed light on this issue. Amphetamine-like substances and synthetic cathinones are usually consumed as a racemic mixture of R-(-)-enantiomer and S-(+)-enantiomer although the excretion products after consumption are often R (or S) enriched mixtures. For substances having stereoselective human metabolism, determining enantiomeric enrichment factor (EF) not equal to 0.5 remarks potential human consumption (Archer et al., 2018; Castrignanò et al., 2018; Kasprzyk-Hordern and Baker, 2012). Castrignanò *et al.* could determine human consumption of mephedrone in Bristol using enantiomeric analysis (Castrignanò et al., 2018).

v. ***Ethical implications.*** Wastewater analysis of large catchment areas does not represent any ethical concern since no single individual or small community (< 10,000 individuals) can be related to the results. However, when analyzing small areas or communities such as night clubs or music festivals, ethical issues might arise and researchers need to be cautious to anonymize results before going public (Hall et al., 2012).

In order to respond to the analytical challenge of monitoring NPS consumption thorough WW analysis, and considering the key topics abovementioned, different analytical approaches have been proposed in the scientific literature as also highlighted in **scientific articles 8** and **10** of this thesis. As a summary, a comprehensive and meaningful strategy should, at least, include the following essential cornerstones:

vi. ***Maintenance of updated NPS databases.*** The high number of new NPS appearing every year, the large amount of potential metabolites associated as well as their rapid transience on the drug market hugely difficult the identification of these substances. Therefore, the development of NPS databases based on surveys, seizures, intoxications

and EWS including analytically related data such as RT, expected precursor and production ions, and/or CCS values is of paramount importance to foster detection of substances (Diamanti et al., 2019; Mardal et al., 2021; Salgueiro-González et al., 2019). However, these databases need to be updated on a regular basis to include new substances of relevance and metabolites to be monitored in order not get easily outdated. Online collaborative databases such as NPS Data Hub or HighRes NPS have been developed in order to facilitate the exchange of information between researchers working on NPS detection (Mardal et al., 2019; Urbas et al., 2018). These databases are fed with mass spectrometric information related to new substances detected by laboratories enabling to expand the screening capacity of contributing laboratories. At the moment of writing this thesis, HighRes NPS contains more than 2,000 unique entries. The implementation of such large databases is pivotal to allow the comprehensive monitor of NPS through WW analysis facilitating the reporting of reliable data with the inclusion of recently reported substances.

vii. ***Targeted analysis.*** The directed analysis towards a list of NPS is the most widely used approach for the monitoring of NPS through WW analysis. In general, targeted analyses are based on NPS databases, NPS prevalence data, or previously detected compounds as is the case of **scientific article 9**. Targeted analysis can be either performed using HRMS instruments (Bade et al., 2019b, 2020c; Bijlsma et al., 2020; Diamanti et al., 2019; Mardal et al., 2021; Salgueiro-González et al., 2019) or LRMS instruments (Bade et al., 2021, 2020b, 2017; Brandeburová et al., 2020; González-Mariño et al., 2018a, 2016b; Kinyua et al., 2015; O'Rourke and Subedi, 2020; Senta et al., 2015) depending on the purpose of the analysis. Generally, HRMS studies compile a larger list of target compounds in order to provide a bigger snapshot of the consumption of NPS (Diamanti et al., 2019; Mardal et al., 2021; Salgueiro-González et al., 2019). However, the usually low concentration of NPS biomarkers in WW samples often requires the utilization of more sensitive and selective instruments. Consequently,

the vast majority of targeted studies rely on LRMS instruments although target lists are often shorter. LRMS targeted analyses can provide lower LOD and LOQ and, therefore, enable the detection of less consumed substances which might represent a benefit compared to HRMS studies. Besides, further efforts using newly developed instrumentation with enhanced sensitivity are needed to improve NPS monitoring in WW and PU. As shown in **scientific article 9**, the improvement in sensitivity provided by μLC represents a step-forward into the detection of low concentrated compounds in WW. Although this technique cannot yet be implemented as a routine strategy because of the low in-matrix reproducibility of the chromatographic separation, it is expected that future developments may allow its utilization for NPS analysis. From a different perspective, targeted analysis allows the optimization of the chromatographic separation enabling a superior overall performance of the strategy. Most studies monitor chemical families with large structural similarities that may result in closely eluting compounds if no chromatographic separation optimization is performed. In this sense, special attention needs to be paid on the separation, so that RT could also be used as an identification parameter. However, one of the main limitation of targeted analysis is the necessity of reference standards of NPS biomarkers.

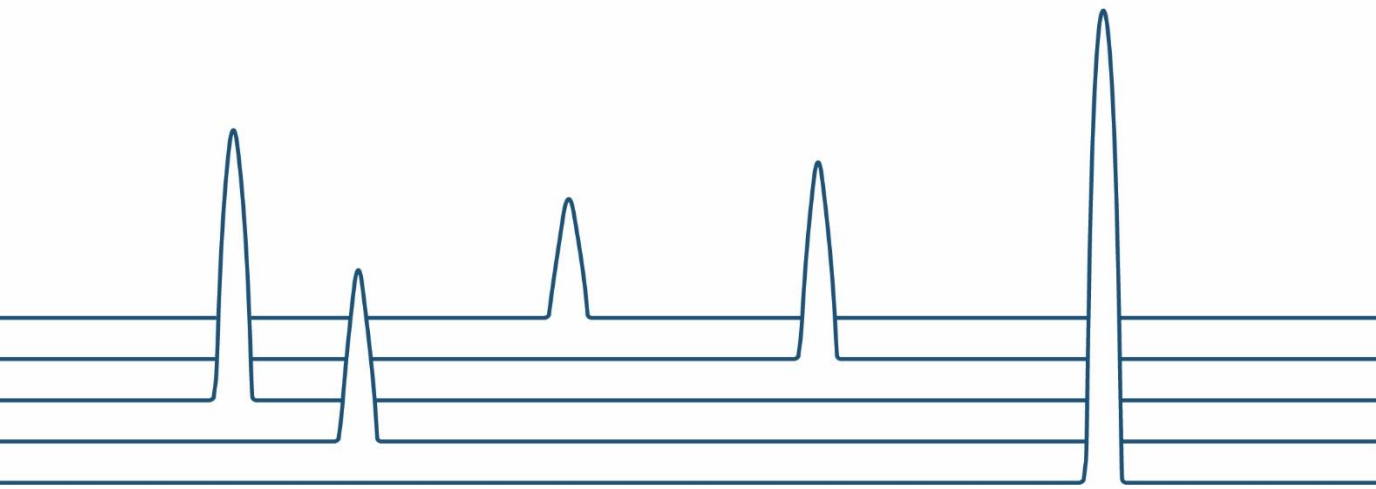
viii. ***Untargeted analysis.*** Untargeted analysis, including suspect and non-target screening, has also been explored in the field of NPS monitoring through WW analysis (Bade et al., 2020c, 2019a, 2019b, 2018; Baz-Lomba et al., 2016; Bijlsma et al., 2020; Diamanti et al., 2019; González-Mariño et al., 2016a; Mardal et al., 2021; Salgueiro-González et al., 2019). Since samples can be analyzed without the necessity of having reference standards prior to analysis, these strategies permit to screen in theory for an unlimited number of substances in a single injection. In this sense, collaborative databases such as HighRes NPS or NPS Data Hub play a key role in providing high quality analytical information for laboratories to implement in their suspect screening analyses. Additionally, full-spectrum data acquired in untargeted mode permits retrospective

analysis in former samples to search for newly discovered substances or metabolites. Even so, detected substances require to purchase their corresponding reference standards for confirmation or the application of predictive tools in the case when no standard is commercially available to gain more confidence in the identification. However, compared to target LRMS strategies, untargeted HRMS approaches are less sensitive and can suffer from extra matrix effects since no chromatographic separation can be optimized. In this sense, the coupling of IMS to HRMS smooths the identification process by removing co-eluting matrix interferences and also providing with an additional identification parameter.

Monitoring NPS through WW and PU analysis is an arduous and challenging task for analytical chemists that requires a well-planned and carefully thought-out strategy. Tackling such a public health issue demands that high-quality data is produced. In this sense, the combination of updated NPS databases, targeted analyses and wide-scope suspect screening strategies using HRMS can provide a comprehensive overview of the NPS scenario.

CHAPTER 6.

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK



6.1. Conclusions

The **general conclusion** of this thesis is that the combination of different strategic workflows (*i.e.* target, suspect and non-target screening) together with different refinement tools such as retention time indexing, *in* silico prediction models or effect-directed analysis is the most powerful approach for the monitoring of small molecules in complex environmental samples. However, the data generated with these approaches is complex and requires that the analyst has a high background knowledge to appropriately curate the data and extract results.

In order to meet this general conclusion, several **specific conclusions** have been drawn. They can be summarized as follows:

i. Liquid chromatography coupled to IMS and QTOF MS is a powerful analytical technique for the identification of OMP in complex matrices. The additional identification parameter provided by CCS values is of additional help for the identification of small molecules.

ii. The extra separation provided by IMS is highly valuable due to the fact that it is able to provide cleaner MS spectra and, thus, facilitate the data revision and compound identification processes. Also, the robustness of

CCS measurement makes it possible to identify a molecule even when RT has been shifted because of matrix interferences.

iii. The inclusion of IMS into the identification criteria helps to reduce the number of false positive detections in automated screening strategies without compromising the number of true identifications. Therefore, the data revision step is facilitated by reducing the number of features that need to be studied and, as a consequence, it increases the analyses throughput.

iv. Retention time indexing is a powerful approach to account for RT deviations due to matrix interferences despite the fact that its application in liquid chromatographic system is not straightforward. Different approaches such as the one proposed in this thesis are laying the basis for widely implemented future models able to deal with the large set of parameters affecting LC retention.

v. *In silico* prediction of CCS by means of ANN is of great help for the analysis of small molecules. In those cases for which no reference standard is available, predictive models can give additional information to better decide which candidate is worthy to invest more time efforts.

vi. Open access *in silico* prediction models such as the one developed by means of MARS for RT and CCS are more applicable for the wider scientific community. A free-access online platform has been released to enable the use of the predictor in third-party laboratories.

vii. Open access databases comprising large lists of compounds as well as online available mass spectral databases are key for the application of wide scope suspect screening strategies.

viii. Water quality assessment cannot only be based on chemical analyses. Biological analyses are able to reflect the overall toxicological activity of the sample and, therefore, evaluate if it might represent a threat for the surrounding ecosystem. In this sense, an appropriate selection of toxicity endpoints is pivotal to obtain meaningful data.

ix. The integration of chemical analysis with biological analysis better reflects the overall status of a water body than the solely application of chemical or biological analyses. Thus, EDA is a highly valuable and

promising strategy that is able to give a complete snapshot of the current state of the assessed water body as well as to identify the toxicity drivers. However, improvements in EDA are still needed before it can be fully incorporated in every research laboratory.

x. For the particular case of NPS monitoring, there are still several challenges to be faced such as the rapid transience of NPS in the drug market, the lack of metabolism data, the low concentrations expected in wastewater samples and the high sensitivity and selectivity required for the reliable determination of NPS in complex matrices.

xi. The reduced amount of wastewater sample needed when developing more sensitive methodologies for the determination of NPS and ID notably facilitates sample collection and shipping in large sampling campaigns.

xii. An outstanding improvement in sensitivity was observed in micro liquid chromatography. However, the robustness of the chromatographic separation was not enough to permit the validation of the methodology.

xiii. Smart sample collection strategies are pivotal to provide meaningful information on NPS use. In this sense, collection of 24h composite WW samples as well as the collection of PU samples from pissoirs at specific settings with an expected higher consumption of NPS can be of great help.

xiv. Although the quantitative determination of NPS in WW and PU samples is vastly reported in the literature; qualitative approaches by means of HRMS are also useful. The latter enable the untargeted search for metabolites and the retrospective screening of previously unknown NPS. Additionally, collaborative NPS mass spectral databases smooth the identification process since third-party empirical data is available to consult.

Overall, the main message that can be derived from this thesis is that collaboration between researchers from different scientific fields is essential. By joining forces, knowledge and experiences with other colleagues, the outcome of

the research efforts is always richer and more fruitful. Thus, new research gaps or problems come into existence and new opportunities for research are opened.

6.1. Conclusiones

La **conclusión general** de esta tesis reside en el hecho de que la combinación de diferentes estrategias (*i.e.* cribado dirigido, de sospechosos y no dirigido) junto con diferentes herramientas de mejora tales como índices de tiempo de retención, modelos computacionales de predicción o el análisis basado en efecto es la aproximación más poderosa para el control de pequeñas moléculas en muestras ambientales. Sin embargo, los datos generados con estas aproximaciones son complejos y requieren un alto grado de conocimiento por parte del analista encargado de curar los datos y extraer resultados.

Para poder llegar a esta conclusión general, se han obtenido previamente algunas **conclusiones específicas**. Estas pueden resumirse en:

i. La cromatografía líquida acoplada a IMS y QTOF MS es una poderosa técnica analítica para la identificación de OMP en matrices complejas. El parámetro de identificación adicional proporcionado por los valores de CCS es de gran ayuda para la identificación de pequeñas moléculas.

ii. La separación adicional obtenida mediante IMS es altamente valiosa debido a que resulta en espectros de MS más limpios y, por tanto, facilita la revisión de los datos y los procesos de identificación de compuestos.

Además, la robustez en la medida de los valores de CCS hace posible la identificación de una molécula aun cuando el RT ha sido desviado por interferentes de la matriz.

iii. La inclusión de IMS en los criterios de identificación ayuda a reducir el número de falsos positivos detectados en estrategias de cribado automatizadas. Así pues, la etapa de revisión de datos es facilitada mediante la reducción del número de detecciones que necesitan estudio y, en consecuencia, incrementa el rendimiento de los análisis.

iv. Los índices de tiempo de retención son una poderosa aproximación para contrarrestar las derivas del RT debidas a la matriz; aunque su aplicación en cromatografía líquida no es sencilla. Diferentes estrategias como la propuesta en esta tesis sientan las bases de futuros modelos ampliamente implementados que sean capaces de lidiar con la gran cantidad de parámetros que afectan la retención en cromatografía líquida.

v. La predicción *in silico* de valores de CCS mediante ANN es de gran utilidad para el análisis de moléculas pequeñas. En los casos en los que el patrón de referencia no esta disponible, los modelos de predicción pueden aportar información adicional que permita decidir qué candidatos merecen la inversión de más tiempo de estudio.

vi. Los modelos de predicción *in silico* de acceso libre como el desarrollado mediante MARS para RT y CCS son más aplicables entre la comunidad científica. Se ha publicado una plataforma para la predicción de CCS y RT de acceso libre.

vii. Las bases de datos de acceso libre que contienen grandes listas de compuestos así como las librerías espectrales disponibles en línea son esenciales para la aplicación de cribados de sospechosos de amplio espectro.

viii. El control de la calidad del agua no puede llevarse a cabo sólo mediante análisis químico. El análisis biológico es capaz de reflejar la toxicidad global de una muestra y, de este modo, evaluar si representa una amenaza para el ecosistema que la rodea. En este sentido, una correcta selección de las variables toxicológicas de interés es esencial.

ix. La integración del análisis químico con el análisis biológico refleja el estado global del medio acuático de una mejor manera que la aplicación independiente de análisis químico o biológico. En este sentido, EDA es una estrategia prometedora y de alto valor capaz de dar una visión general de la toxicidad así como de identificar los compuestos responsables de dicha toxicidad. Sin embargo, son necesarias todavía algunas mejoras que permitan incorporar plenamente EDA en cada laboratorio de investigación.

x. Para el caso particular del control de NPS, todavía existen diversos retos que deben superarse como, por ejemplo, la rápida transitoriedad de los NPS en el mercado, la falta de datos acerca del metabolismo de las sustancias, las bajas concentraciones esperadas en las aguas residuales y la alta sensibilidad y selectividad necesarias para una correcta identificación.

xi. La reducción en el volumen de muestra de aguas residuales necesario durante la aplicación de metodologías altamente sensibles para la determinación de ID y NPS facilita notablemente la recolección de las muestras y su transporte en grandes campañas de muestreo.

xii. Se observó un extraordinario aumento en la sensibilidad proporcionada por cromatografía micro fluídica. Sin embargo, la robustez de la separación cromatográfica no fue suficiente para permitir la validación del método.

xiii. Las estrategias de recolección de muestra inteligentes son clave para proveer de información valioso acerca del consumo de NPS. En este sentido, son de gran utilidad la recolección de muestras de aguas residuales compuesta de 24-h o la recolección de muestras de PU de urinarios en lugares específicos con un previsible mayor consumo de NPS.

xiv. Aunque la determinación cuantitativa de NPS en WW y PU ha sido ampliamente reportada en la literatura, las estrategias cualitativas mediante HRMS también son de utilidad. Estas permiten la búsqueda no dirigida de metabolitos, así como el análisis retrospectivo de NPS previamente desconocidos. Además, existen bases de datos

colaborativas que facilitan el proceso identificativo mediante datos empíricos de otros laboratorios.

En general, el mensaje principal de esta tesis también reside en que la colaboración entre investigadores de distintos campos científicos es primordial. Los resultados obtenidos tras el esfuerzo analítico siempre son más ricos y fructíferos mediante la unión de fuerzas, conocimiento y experiencias con otros colegas. De este modo, se abren nuevos nichos de investigación y, por tanto, nuevas oportunidades de estudio.

6.2. Suggestions for future research

This thesis has demonstrated the strong potential of UHPLC-IMS-HRMS for the monitoring of small molecules in complex matrices, especially when performed in combination with *in silico* prediction tools and biological analysis. From the results and conclusions gathered in the studies herein presented, future research lines can be suggested.

The main topics to be addressed in the forthcoming years can be summarized as follows:

i. Expand the availability of open access databases featuring IMS data for OMP. Additionally, explore and evaluate the inter-comparability of CCS values obtained with different instrumental technologies such as TWIMS or DTIMS, so that online available data can be of use regardless the instrument used.

ii. Further investigate the role of IMS in the separation of isomers and protomers. It is expected that improvements in IMS technologies will allow the separation of isomers in routine analyses.

iii. Contribute to a better performance of *in silico* prediction models by developing more accurate predictive tools. Explore the applicability of prediction models to third-party instrumental configurations.

iv. Investigate the potential and possibilities of EDA for the assessment of water quality. Implement fractionation steps in the EDA workflow in order to facilitate the identification of toxicity drivers in environmental samples.

v. Contribute to gather more knowledge on the actual extent of NPS use by participating in large wastewater sampling campaigns at both national and international levels.

6.2. Sugerencias de trabajo futuro

Esta tesis ha demostrado el fuerte potencial de UHPLC-IMS-HRMS para el control de moléculas pequeñas en matrices complejas, especialmente cuando se lleva a cabo en combinación con modelos predictivos y análisis biológico. A partir de estos resultados y conclusiones, se pueden sugerir diferentes líneas de investigación futuras.

Los principales temas que necesitarán ser resueltos en los próximos años se pueden resumir de este modo:

i. Ampliar la disponibilidad de bases de datos de acceso abierto que incluyan información de IMS para OMP. Además, explorar y evaluar la comparabilidad de los valores de CCS obtenidos con diferentes tecnologías instrumentales como TWIMS o DTIMS, de tal modo que los datos disponibles en línea puedan utilizarse independientemente del instrumento en que fueron adquiridos.

ii. Investigar en profundidad el papel de IMS en la separación de isómeros y protómeros. Es esperable que futuras mejoras en las tecnologías de IMS permitan la separación de estos compuestos en análisis de rutina.

iii. Contribuir a una mejor actuación de los modelos computacionales predictivos mediante el desarrollo de herramientas predictivas más precisas. Explorar la aplicabilidad de estos modelos a otras configuraciones instrumentales.

iv. Investigar el potencial y las posibilidades de EDA para la evaluación de la calidad de lagua. Implementar etapas de fraccionamiento en el flujo de trabajo de EDA para facilitar la identificación de los responsables de la toxicidad en muestras ambientales.

v. Contribuir a generar mayor conocimiento sobre el alcance real del consumo de NPS mediante la participación en amplias campañas de muestreo de aguas residuales tanto a nivel nacional como internacional.

CHAPTER 7.

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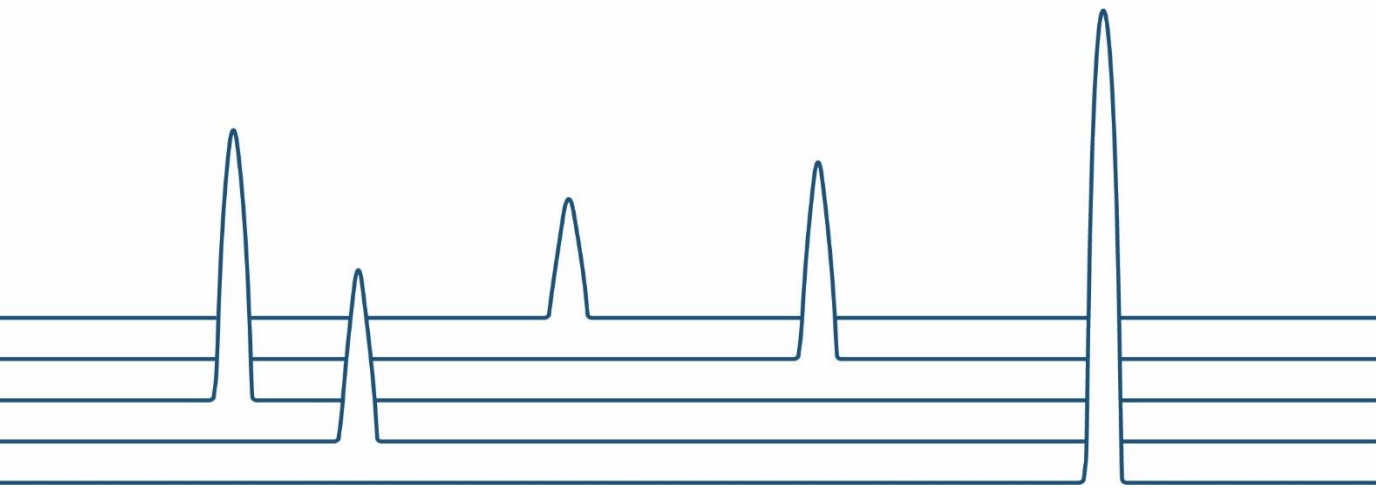

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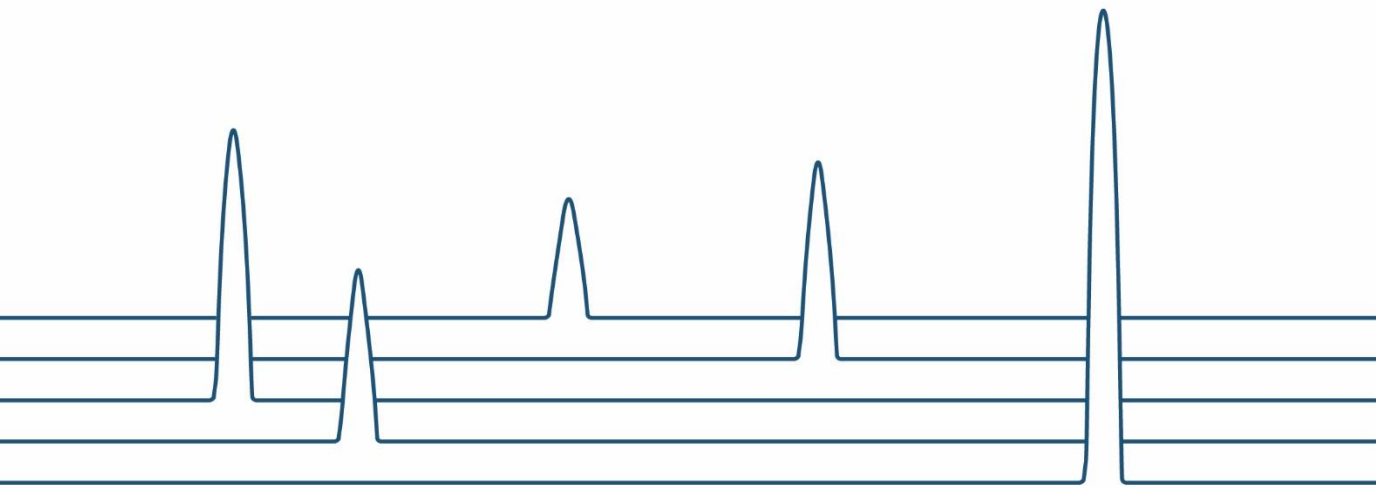
ANNEX I.

CO-AUTHOR AGREEMENT



UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana

17th June 2021

I, **Lubertus Bijlsma**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

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In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

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- "Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations". Lubertus Bijlsma*, **Alberto Celma***, Francisco López, Félix Hernández. *Current Opinion in Environmental Science & Health*, 9 (2019), 1-12. DOI: 10.1016/j.coesh.2019.03.002. (* Co-first authors)

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- "Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial". Lubertus Bijlsma, Richard Bade, Frederic Been, **Alberto Celma**, Sara Castiglioni. *Analytica Chimica Acta*, 1145 (2021), 132-147. DOI: 10.1016/j.aca.2020.08.058.

Signed, **Lubertus Bijlsma**

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Signed, **Juan Vicente Sancho Llopis**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana

17th June 2021

I, **Félix Hernández Hernández**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

- "Prediction of Collision Cross Section Values for Small Molecules: Application to Pesticide Residue Analysis". Lubertus Biljsma*, Richard Bade*, **Alberto Celma**, Lauren Mullin, Gareth Cleland, Sara Stead, Félix Hernández, and Juan V. Sancho. *Analytical Chemistry*, 89 12 (2017), 6583-6589. DOI: 10.1021/acs.analchem.7b00741. (* Co-first authors)

- "Dual prediction of retention time and collision cross section of emerging organic contaminants for environmental analyses". **Alberto Celma***, Richard Bade*, Melissa Humphries, Juan V. Sancho, Félix Hernández and Lubertus Bijlsma. *Analytical Chemistry*, (2021), Submitted. (*Co-first authors).

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

- "Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations". Lubertus Bijlsma*, **Alberto Celma***, Francisco López, Félix Hernández. *Current Opinion in Environmental Science & Health*, 9 (2019), 1-12. DOI: 10.1016/j.coesh.2019.03.002. (* Co-first authors)

- "Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology". **Alberto Celma**, Juan V. Sancho, Noelia Salgueiro-González, Sara Castiglioni, Ettore Zuccato, Félix Hernández and Lubertus Bijlsma. *Journal of Chromatography A*, 1602 (2019), 300-309. DOI: 10.1016/j.chroma.2018.07.030.

Signed, **Félix Hernández**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

16th June 2021

I, **Lutz Ahrens**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

Signed, **Lutz Ahrens**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Brisbane, Australia

15th June 2021

I, **Richard Bade**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Prediction of Collision Cross Section Values for Small Molecules: Application to Pesticide Residue Analysis". Lubertus Biljsma*, Richard Bade*, **Alberto Celma**, Lauren Mullin, Gareth Cleland, Sara Stead, Félix Hernández, and Juan V. Sancho. *Analytical Chemistry*, 89 12 (2017), 6583-6589. DOI: 10.1021/acs.analchem.7b00741. (* Co-first authors)
- "Dual prediction of retention time and collision cross section of emerging organic contaminants for environmental analyses". **Alberto Celma***, Richard Bade*, Melissa Humphries, Juan V. Sancho, Félix Hernández and Lubertus Bijlsma. *Analytical Chemistry*, (2021), Submitted. (*Co-first authors).
- "Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial". Lubertus Bijlsma, Richard Bade, Frederic Been, **Alberto Celma**, Sara Castiglioni. *Analytica Chimica Acta*, 1145 (2021), 132-147. DOI: 10.1016/j.aca.2020.08.058.

Signed, **Richard Bade**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Wilmslow, United Kingdom

17th May 2021

I, **Gitte Barknowitz**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

Signed, **Gitte Barknowitz**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Nieuwegein, The Netherlands

17th May 2021

I, **Frederic Been**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial". Lubertus Bijlsma, Richard Bade, Frederic Been, **Alberto Celma**, Sara Castiglioni. *Analytica Chimica Acta*, 1145 (2021), 132-147. DOI: 10.1016/j.aca.2020.08.058.

Signed, **Frederic Been**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Milan, Italy

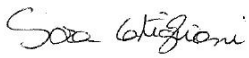
20th May 2021

I, **Sara Castiglioni**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology". **Alberto Celma**, Juan V. Sancho, Noelia Salgueiro-González, Sara Castiglioni, Ettore Zuccato, Félix Hernández and Lubertus Bijlsma. *Journal of Chromatography A*, 1602 (2019), 300-309. DOI: 10.1016/j.chroma.2018.07.030.

- "Perspectives and challenges associated with the determination of new psychoactive substances in urine and wastewater – A tutorial". Lubertus Bijlsma, Richard Bade, Frederic Been, **Alberto Celma**, Sara Castiglioni. *Analytica Chimica Acta*, 1145 (2021), 132-147. DOI: 10.1016/j.aca.2020.08.058.



Signed, **Sara Castiglioni**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Milford, United States

17th May 2021

I, **Gareth Cleland**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Prediction of Collision Cross Section Values for Small Molecules: Application to Pesticide Residue Analysis". Lubertus Biljsma*, Richard Bade*, **Alberto Celma**, Lauren Mullin, Gareth Cleland, Sara Stead, Félix Hernández, and Juan V. Sancho. *Analytical Chemistry*, 89 12 (2017), 6583-6589. DOI: 10.1021/acs.analchem.7b00741. (* Co-first authors)

Signed, **Gareth Cleland**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana

19th May 2021

I, **David Fabregat Safont**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

Signed, **David Fabregat Safont**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Barcelona

16th June 2021

I, **Pablo Gago Ferrero**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

GAGO FERRERO, PABLO (AUTENTICACIÓN)

Digitally signed by GAGO FERRERO, PABLO (AUTENTICACIÓN)
DN: c=ES, serialNumber=45786547X, sn=GAGO, givenName=PABLO, cn=GAGO FERRERO, PABLO (AUTENTICACIÓN)
Date: 2021.06.17 10:15:06 +02'00'

Signed, **Pablo Gago Ferrero**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

16th June 2021

I, **Oksana Golovko**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

Signed, **Oksana Golovko**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Manchester, United Kingdom

19th May 2021

I, **Jeff Goshawk**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

Signed, **Jeff Goshawk**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Adelaide, Australia

15th June 2021

I, **Melissa Humphries**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Dual prediction of retention time and collision cross section of emerging organic contaminants for environmental analyses". **Alberto Celma***, Richard Bade*, Melissa Humphries, Juan V. Sancho, Félix Hernández and Lubertus Bijlsma. *Analytical Chemistry*, (2021), Submitted. (*Co-first authors).

Signed, **Melissa Humphries**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana

19th May 2021

I, **María Ibáñez Martínez**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

Signed, **María Ibáñez Martínez**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

16th June 2021

I, **Foon Yin Lai**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

Signed, **Foon Yin Lai**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana

19th May 2021

I, **Francisco López Benet**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

- "Development of a Retention Time Interpolation scale (RTi) for liquid chromatography coupled to mass spectrometry in both positive and negative ionization modes". **Alberto Celma**, Lubertus Bijlsma, Francisco López, Juan V. Sancho. *Journal of Chromatography A*, 1568 (2018), 101-107. DOI: 10.1016/j.chroma.2018.07.030.

- "Monitoring new psychoactive substances use through wastewater analysis: current situation, challenges and limitations". Lubertus Bijlsma*, **Alberto Celma***, Francisco López, Félix Hernández. *Current Opinion in Environmental Science & Health*, 9 (2019), 1-12. DOI: 10.1016/j.coesh.2019.03.002. (* Co-first authors)

Signed, **Francisco López Benet**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

21st June 2021

I, **Johan Lundqvist**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

- "In vitro bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline". **Alberto Celma**, Geeta Mandava, Agneta Oskarsson, Juan Vicente Sancho, Lubertus Bijlsma, Johan Lundqvist. *Environmental Sciences Europe* 33:70 (2021) 1-12. DOI: 10.1186/s12302-021-00510-1.

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

Signed, **Johan Lundqvist**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

15th June 2021

I, **Geeta Mandava**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "In vitro bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline". **Alberto Celma**, Geeta Mandava, Agneta Oskarsson, Juan Vicente Sancho, Lubertus Bijlsma, Johan Lundqvist. *Environmental Sciences Europe* 33:70 (2021) 1-12. DOI: 10.1186/s12302-021-00510-1.

Signed, **Geeta Mandava**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

16th June 2021

I, **Frank Menger**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

Signed, **Frank Menger**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Milford, United States

17th May 2021

I, **Lauren Mullin**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Prediction of Collision Cross Section Values for Small Molecules: Application to Pesticide Residue Analysis". Lubertus Biljsma*, Richard Bade*, **Alberto Celma**, Lauren Mullin, Gareth Cleland, Sara Stead, Félix Hernández, and Juan V. Sancho. *Analytical Chemistry*, 89 12 (2017), 6583-6589. DOI: 10.1021/acs.analchem.7b00741. (* Co-first authors)

25 May 2021

Signed, **Lauren Mullin**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

15th June 2021

I, **Agneta Oskarsson**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "In vitro bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline". **Alberto Celma**, Geeta Mandava, Agneta Oskarsson, Juan Vicente Sancho, Lubertus Bijlsma, Johan Lundqvist. *Environmental Sciences Europe* 33:70 (2021) 1-12. DOI: 10.1186/s12302-021-00510-1.

Signed, **Agneta Oskarsson**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Castelló de la Plana

19th May 2021

I, **Elena Pitarch Arquimbau**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

Signed, **Elena Pitarch Arquimbau**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Milan, Italy

17th May 2021

I, **Noelia Salgueiro González**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology". **Alberto Celma**, Juan V. Sancho, Noelia Salgueiro-González, Sara Castiglioni, Ettore Zuccato, Félix Hernández and Lubertus Bijlsma. *Journal of Chromatography A*, 1602 (2019), 300-309. DOI: 10.1016/j.chroma.2018.07.030.

Signed, **Noelia Salgueiro González**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Luxembourg

19th May 2021

I, **Emma L. Schymanski**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Improving Target and Suspect Screening High-Resolution Mass Spectrometry Workflows in environmental analysis by Ion Mobility Separation". **Alberto Celma**, Juan V. Sancho, Emma L. Schymanski, David Fabregat-Safont, María Ibáñez, Jeff Goshawk, Gitte Barknowitz, Félix Hernández and Lubertus Bijlsma. *Environmental Science and Technology*, 53, 23 (2020), 15120-15131. DOI: 10.1021/acs.est.0c05713.

Signed, **Emma L. Schymanski**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

Manchester, United Kindom

8th June 2021

I, **Sara Stead**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Prediction of Collision Cross Section Values for Small Molecules: Application to Pesticide Residue Analysis". Lubertus Biljsma*, Richard Bade*, **Alberto Celma**, Lauren Mullin, Gareth Cleland, Sara Stead, Félix Hernández, and Juan V. Sancho. *Analytical Chemistry*, 89 12 (2017), 6583-6589. DOI: 10.1021/acs.analchem.7b00741. (* Co-first authors)

Signed, **Sara Stead**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
4. In the case of joint publications, all the co-authors must explicitly state their approval that the doctoral student presented the work as part of her/his thesis and the express waiver of presenting this same work as part of another doctoral thesis. This authorisation must be attached as documentation when the evaluation of the thesis begins."

UNIVERSITAT JAUME I
Escola de Doctorat · ED

Uppsala, Sweden

16th June 2021

I, **Karin Wiberg**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "The relevant role of ion mobility separation in LC-HRMS based screening strategies for contaminants of emerging concern in the aquatic environment". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Félix Hernández, Francisco López, Johan Lundqvist, Elena Pitarch, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Chemosphere*, 208 (2021), 130799. DOI: 10.1016/j.chemosphere.2021.130799.

- "Integration of chemical analysis and bioanalysis for a comprehensive water quality evaluation in Spanish Mediterranean coastline water bodies". **Alberto Celma**, Lutz Ahrens, Pablo Gago-Ferrero, Oksana Golovko, Félix Hernández, Foon Yin Lai, Johan Lundqvist, Frank Menger, Juan V. Sancho, Karin Wiberg, Lubertus Bijlsma. *Water Research* (2021) Submitted.

Signed, **Karin Wiberg**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
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UNIVERSITAT JAUME I
Escola de Doctorat · ED

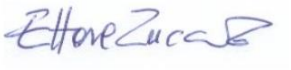
Milan, Italy

17th May 2021

I, **Ettore Zuccato**, hereby authorise **Alberto Celma Tirado** to include the publications listed below in his doctoral thesis. In addition, I waive the right to use those articles as part of any other doctoral thesis.

List of articles:

- "Simultaneous determination of new psychoactive substances and illicit drugs in sewage: Potential of micro-liquid chromatography tandem mass spectrometry in wastewater-based epidemiology". **Alberto Celma**, Juan V. Sancho, Noelia Salgueiro-González, Sara Castiglioni, Ettore Zuccato, Félix Hernández and Lubertus Bijlsma. *Journal of Chromatography A*, 1602 (2019), 300-309. DOI: 10.1016/j.chroma.2018.07.030.



Signed, **Ettore Zuccato**

In accordance with article 28 of the Regulations on doctoral studies of the Universitat Jaume I in Castelló, *regulated by RD 99/2011, at the Universitat Jaume I (*Approved by the Governing Council at its meeting no. 8/2020 held on 2 October 2020*):*
"(...)
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